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14. ABSTRACT

Basal-like breast cancers (BLBCs) generally become resistant to cytotoxic agents and resistance has been attributed to the presence of tumor initiating cancer stem cells (CSCs). Furthermore, LRP6/Wnt appears to play a crucial role in BLBC and CSC progression, and may represent an excellent therapeutic target. We have previously described that TRA-8, a monoclonal antibody specific to death receptor 5, kills both the CSCs and non-CSC population of BLBCs. This study examined two questions: whether niclosamide (an FDA approved antihelminthic, that inhibits Wnt/β-catenin signaling) is cytotoxic to BLBCs and its CSC population; and whether niclosamide in combination with TRA-8 produces synergistic cytotoxicity. We characterized non-adherent ALDH enriched (NAAE) cells as a CSC enriched population from BLBC cell lines. Both Adherent and NAAE cells from 2LMP, SUM159, HCC1187, HCC1143 cell lines and patient pleural effusion samples showed that niclosamide inhibited Wnt/β-catenin pathway activation, down regulated LRP6, and decreased downstream β-catenin signaling. The combination of TRA-8 and niclosamide showed additive to synergistic cytotoxicity and further reduced Wnt/β-catenin activity. In vivo studies also showed that intraperitoneal administration of niclosamide in combination with TRA-8 suppressed growth of established 2LMP orthotopic tumor xenografts. Treatment with niclosamide in combination with TRA-8 may be an effective therapy against BLBC.

15. SUBJECT TERMS
Breast cancer stem cells, tumor initiating cells, TRA-8, Death receptor 5, Adriamycin, Taxol, Wnt

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Introduction

Basal-Like Breast Cancer (BLBC) accounts for 13% of all breast carcinomas [1]. It is characterized by a unique mRNA profile with CK5/6 expression, inactivation of *BRCA1* and lack of estrogen receptor and HER-2 amplification [1-3]. BLBC is considered one of the most aggressive, metastatic, and chemoresistant breast cancer subtypes [4]. Its poor prognosis is linked to enrichment for tumor initiating cancer stem cells (CSC) [5]. The *Cancer Stem Cell Model* suggests that tumors, similar to normal tissue, are organized in a cellular hierarchy, with (CSC) at the top, as the cells with potentially limitless proliferation abilities [6]. The more 'differentiated' descendants, which account for the majority of the tumor population, may also be able to proliferate, but regenerative ability is limited [6]. Traditional chemotherapy agents target these differentiated cells, but unfortunately fail to kill the stem cell progenitor population [7]. The chemoresistant stem cells are thought to be responsible for relapse and metastasis of many tumor types [7]. Several investigators have shown that BLBC cell lines and patient samples contain a subpopulation of breast cancer stem cells (BrCSC) [8, 9]. These BrCSC are identified based on their enhanced tumorigenicity, tumorsphere forming ability, expression of $CD44^{+}/CD24^{-}$, elevated enzymatic activity of aldehyde dehydrogenase (ALDH), and dysregulation of self-renewing pathways, including Wnt, Hedgehog, and Notch signaling [10, 11]. BrCSC also overexpress ABC efflux transporters, detoxification enzymes, and have slower turnover rate that make this sub-population likely to become resistant to chemotherapy [12, 13]. Thus an effective BLBC therapeutic strategy must kill these propagating and chemoresistant BrCSC in addition to the proliferating non-stem cell cancer population [14].

One promising approach to prevent BLBC recurrence and metastasis is to target pathways that regulate tumor initiation such as the Wnt/β-catenin pathway [4, 15]. A cell surface receptor called LRP6, essential for Wnt/β-catenin signaling, is a potential target because its expression is frequently up-regulated in 20-36% of human breast cancers. Furthermore, suppression of LRP6 is sufficient to inhibit Wnt signaling in breast cancer [16]. Specifically in BLBC, LRP6 is expressed at elevated levels not seen in any other breast cancer subtype, thus providing an excellent potential target for treatment [15-18]. The Wnt/β-catenin pathway can be inhibited by Niclosamide, which degrades LRP6 and interacts with Frizzled thus preventing proliferation and causing apoptosis [19-21]. Niclosamide (trade name Niclocide) is a teniacide in the antihelminth family that has been FDA approved for the treatment of tapeworms and has been used in humans for nearly 50 years [22-24]. Niclosamide has also been shown to be cytotoxic against prostate and colorectal cancer, myelogenous leukemia, and in ovarian cancer it suppresses CSC and metastasis [21, 25-27]. TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) also inhibits Wnt/β-catenin signaling by promoting caspase 3 and 8 mediated cleavage of β -catenin [28, 29]. TRAIL also induces apoptosis in BLBC, however, these promising aspects of TRAIL have been mitigated by the hepatic cytotoxicity seen with some TRAIL preparations [30, 31]. TRA-8 is a non-hepatotoxic agonistic monoclonal antibody (mAb) to TRAIL death receptor 5 (DR5) [32, 33]. BLBC, are preferentially sensitive to TRA-8 induced apoptosis; however, some BLBC cells lines are resistant to treatment [34]. My hypothesis is that suppression of Wnt activity by Niclosamide will further sensitize BLBC and its CSC population to treatment with TRA-8.

Specific Aims

Aim 1. To isolate $CD44^+/CD24^-/ALDEFLUOR^+$ CSC from established human breast cancer cell lines and patient plural effusion samples and evaluate their functional characteristics such as tumorigenicity, tumorsphere formation and chemoresistance.

Aim 2. To evaluate *in vitro* and *in vivo* induction of apoptosis by anti-DR5 monoclonal antibody in BrCSC isolated from cell lines.

Aim 3. To evaluate *in vitro* induction of apoptosis by anti-DR5 monoclonal antibody in BrCSC isolated from cell lines and patient samples in combination with the Wnt inhibitor, Niclosamide.

Aim 4. To evaluate the *in vivo* therapeutic efficacy of anti-DR5 in combination with Niclosamide in xenograft models.

Body

Aim #1:

Task 1. To isolate $CD44^+/CD24^-/ALDEFLUOR^+$ CSC from established human breast cancer cell lines and patient plural effusion samples and evaluate their functional characteristics such as tumorigenicity, tumorsphere formation and chemoresistance.

Task 1a. Analyze sorted BrCSC population for retention of BrCSC markers (months 1-5)

Task 1b. Functional BrCSC assays (months 6-10)

Task 1a and 1b have been completed. Detailed results were reported in the 2011 annual report. The summary of previous results pertinent for this report are the identification and characterization of non-adherent ALDH enriched (NAAE) cells from BLBC cell lines. NAAE cells were identified from freshly formed tumorspheres and were compared to adherent cells that were sorted for the CSC marker, $ALDH^+$, at 6, 12 and 24 h. From this, NAAE cells were identified within the newly formed tumorspheres. NAAE cells showed 70-80% enrichment for ALDH activity at the 12 h time point compared to sorted $ALDH^+$ cells. In the 2LMP, SUM159, and HCC1143 cell lines, ALDH activity was lost over time for both sorted and NAAE cells (Fig. 1A). NAAE cells (obtained from the tumorspheres at the 12 h time point) showed an enhanced ability to form tumors after mammary fat pad injection (MFP) injection of 20,000 cells in NOD/SCID mice compared to non-adherent cells (obtained from tumorspheres after they had been in non-adherent culture for 4 days). The NAAE cells formed aggressive large tumors faster then the non-adherent cells (Fig. 1B) ($p=0.01$). NAAE cells were generated as a tool to analyze the effect of drugs on CSC enriched populations. Because CSCs represent a small fraction of the total population, it is difficult to run mechanistic studies with these cells especially by techniques such as Western blot and TOPflash reporter assay that require a large number of cells. The isolation of NAAE cells allowed us to test these novel drug combinations on a larger scale. Further studies need to be conducted to better understand what epigenetic alterations such as EMT/MET are occurring to enrich for this transient CSC population [35]. Non-adherent cells alone do not represent an aggressive population. Also, patient pleural effusion samples were characterized by immunohistochemistry for positive Moc31 staining (adenocarcinoma) and negative Calretinin staining (mesothelial cells) (Figure 2).

Aim #2:
To evaluate *in vitro* and *in vivo* induction of apoptosis by anti-DR5 monoclonal antibody in BrCSC isolated from cell lines.

Aim 2 has been completed. Detailed results were reported in the 2011 annual report and in a published manuscript [36]. In summary, doubly enriched BrCSCs (CD44+, CD24-, ALDH+) were exposed to TRA-8 and control reagents and examined for cytotoxicity, caspase activation, tumorsphere formation and tumorigenicity. Doubly enriched BrCSC populations expressed cell surface DR5 and were sensitive to TRA-8 mediated cytotoxicity with induction of caspase 8 and 3 activation. TRA-8 at subnanomolar concentrations inhibited 2LMP and SUM159 BrCSC tumorsphere formation and was more than 50-fold more inhibitory than TRAIL or anti-DR4 at equimolar concentrations. Chemotherapy treatment of 2LMP and SUM159 cell lines resulted in a relative increase of BrCSCs, whereas TRA-8 produced a decrease in the percentage of BrCSCs. TRA-8 exposure to 2LMP and SUM159 BrCSC preparations produced significant inhibition of tumorigenicity.

***Aim #3*:**
To evaluate *in vitro* induction of apoptosis by anti-DR5 monoclonal antibody in BrCSC isolated from cell lines and patient samples, alone and in combination with the Wnt inhibitor, Niclosamide.

Task 3a. Evaluate Niclosamide cytotoxicity in BLBC
NAAE adherent cell lines and patient pleural effusion samples were treated with Niclosamide for 48 h. NAAE cells responded to a lower dose range (0.11-0.35 μM) of Niclosamide treatment compared to adherent cells (0.31-0.54 μM). However, both NAAE and adherent cells had IC_{50} values of 1 μM or less. Three of four NAAE cell lines (SUM159, HCC1187 and HCC1143 but not 2LMP) had significantly lower IC_{50} values compared to the adherent parental cell lines (p-values = 0.015, 0.0004 and 0.0002) (Table 1). MCF10A nonmalignant mammary epithelial cells did not show significant sensitivity to 48 h treatment with Niclosamide (Fig. 3). All four patient pleural effusion samples showed greater then 50% reduction in viability. Patient sample UAB05 did not show a dose response past 50% and patient sample UAB04 reached 70% reduction at the lowest dose but also did not show a dose response. UAB03 and UAB01 both reached a 70% reduction in viability with the lowest does of 1 μM and showed a dose response with two-fold increasing concentrations of Niclosamide (Fig. 4C).

Task 3b. Niclosamide inhibition of Wnt/ß-catenin in BLBC and NAAE cells
Active Wnt/β-catenin signaling causes increased activation of TCF/LEF transcription factors. To confirm the inhibitory effect of Niclosamide on the Wnt/β-catenin pathway, we performed the signaling TOPflash luciferase reporter assay to test for inhibition of Wnt/β-catenin activity. 2LMP, SUM159, HCC1187 and HCC1143 adherent cells where transiently transfected with TOPflash and treated with Niclosamide as shown in Figure 4A. Niclosamide significantly blocked Wnt/β-catenin activity at levels greater then 60% in 2LMP, SUM159 and HCC1143 cell lines (p values = 0.0005, 0.002, 0.006). HCC1187 Wnt/β-catenin TOPflash activity was only significantly inhibited in the presence of Wnt3A ligand (data not shown). Inhibition of Wnt/β-catenin was confirmed by Western blot analysis by examining the total levels of Wnt co-receptor LRP6, phosphorylated LRP6, free β-catenin, total β-catenin and Axin2 after 24 h treatment with Niclosamide (0.5, 0.25, 0.125 μM) on 2LMP adherent and NAAE cells (Fig. 4B). As shown in

Figure 2B, increasing doses of Niclosamide reduced total LRP6 and p-LRP6 expression in both adherent and NAAE cells for both 2LMP and HCC1187 cell lines. There was a dose-dependent reduction in the expression of total and free β-catenin with increasing levels of Niclosamide. Total and free β-catenin expression was inhibited in NAAE cells with a 0.25 μM concentration of Niclosamide. Axin2 is a specific transcriptional target of the Wnt/β-catenin signaling pathway [37]. Increasing doses of Niclosamide in both adherent and NAAE cells caused a reduction in Axin2. Niclosamide also showed inhibition of the Wnt/β-catenin pathway in patient sample UAB03 (Fig. 4D).

Task 3c. *In vitro* combination treatment with TRA-8 and Niclosamide

All 4 BLBC cell lines showed inhibition of secondary tumorsphere formation, with Niclosamide concentrations resulting in half the maximal dose required for tumorsphere inhibition (IT_{50}) of 0.25 μM for 2LMP, 0.09 μM for HCC1143, and 0.175 μM for both HCC1187 and SUM159 (Fig. 5A). Pre-treatment with Niclosamide followed by treatment with TRA-8 resulted in increased inhibition of secondary tumorsphere formation. Niclosamide and TRA-8 combined inhibited 80-90% of secondary tumorsphere formation relative to an average of 60% by Niclosamide alone and 40% by TRA-8 as single agent (p-value < 0.00007) (Fig. 5B). Treatment with Niclosamide alone disrupted tumorsphere formation, with the most significant amount of disruption seen in the HCC1143 cell line (Fig. 5C). Adherent 2LMP, SUM159, HCC1143 and HCC1187 cells were treated with Niclosamide and TRA-8 for 24 h and this resulted in decreased Wnt/β-catenin activity compared to untreated cells. For the 2LMP, SUM159 and HCC1143 cell lines, either agent alone produced greater then 50% reduction in TOPflash activity and the combination resulted in significantly greater reduction compared to Niclosamide treatment alone. The HCC1187 cell line treated with 0.25 ng/mL TRA-8 and 0.25 μM Niclosamide, only showed significant inhibition of Wnt activity compared to untreated controls (Fig. 6).

Task 3d. Confirm and further elucidate the mechanism of anti-Wnt and anti-DR5 pathway interaction. Develop shRNA LRP6 knockdown of 2LMP and HCC1187. Observe if shRNA knockdown of LRP6 in cell lines produces enhanced sensitivity to TRA-8 treatment. Investigate expression of Survivin and death receptor 5 after treatment with Niclosamide. Detect by Western-Blot analysis β-catenin degradation after treatment with TRA-8. Western blot for expression of Wnt/β-catenin signaling proteins in 2LMP LRP6 KD cells compared to shRNA control cells showed reduced LRP6 expression and enhanced sensitivity to TRA-8**.**

Aim #4:

To evaluate the *in vivo* therapeutic efficacy of anti-DR5 in combination with Niclosamide in xenograft models.

Task 4a. *Ex vivo*

Effect of *ex vivo* treatment of BrCSC enriched cells on tumorigenicity in NOD/SCID mice. 2LMP and SUM159 tumorspheres were treated with TRA-8, Niclosamide, or the combination and IgG control antibody for 3 h and implanted into the MFP of groups of five NOD/SCID mice. Table 2 represents the frequency of tumor engraftment and the average tumor size of the tumors that developed. In the 2LMP cell line 4 of 5 small, slow growing tumors were observed to develop within 44 days with TRA-8 and Niclosamide treated cells while 5 of 5 large tumors developed in the IgG control group. With the SUM159 cell line, 3 of 5 tumors developed in the

control group, 1 of 5 in the TRA-8 group, 2 of 5 in the Niclosamide treated group, and 0 of 5 tumors developed in the combination group at 46 days after implantation.

Task 4b. *In vivo* treatment with TRA-8 and Niclosamide
Niclosamide in combination with TRA-8 inhibited 2LMP tumor growth *in vivo*. Synergistic cytotoxicity was observed *in vitro* suggesting that the combination would suppress tumor growth *in vivo*. To test this hypothesis, 4-week-old female athymic nude mice were injected in the MFP with $2x10^6$ 2LMP cells. After a 7-day period, tumors reached 16 mm^2 average size and mice were treated intraperitoneal with TRA-8 (200 ng) and or Niclosamide (12.5 mg/kg). As shown in Figure 7, Niclosamide did not inhibit tumor growth at this dose but when administered in combination with TRA-8 there was a significantly reduced tumor size (p-value = 0.001).

Task 4c. *In vivo* treatment using various doses of Niclosamide in 2LMP BLBC orthotopic tumor animal models. Tumors were established in athymic nude mice by MFP implantation of 2×10^6 2LMP cells. The therapy started when tumors reached a size of 16 mm^2. Niclosamide (30 mg/kg) was given IP for 21 days, TRA-8 (200 μg) was given IP 2x weekly for 3 weeks. Tumor size was measured with calipers twice a week. Each point in the curve represents the mean ± SE (n=5). Single agent niclosamide or TRA-8 vs. control (*$P < 0.05$), combination treatment vs. control (**$P < 0.01$), combination treatment vs. TRA-8 or niclosamide (#$P < 0.05$).

Key research accomplishments:
- BrCSC marker expression is maintained for ~24 h after sorting and cells grown in mammosphere media/low attachment plates acquire elevated levels of ALDH activity by 12 h. This transient population was named non-adherent tumorsphere enriched (NAAE).
- TRA-8 is not cytotoxic to normal breast cells or breast stem cells.
- BrCSC enriched populations have DR5 expression similar to parental cells.
- TRA-8 treatment of basal-like BrCSC populations activates caspase 8 and 3.
- TRA-8 treatment of BrCSC populations dramatically inhibits tumorsphere formation.
- BrCSC from 5 out of 8 cell lines were significantly more sensitive to TRA-8 then parental cells.
- Tumorigenicity of BrCSC was inhibited by treatment with TRA-8.
- Adherent and NAAE cells from 2LMP, SUM159, HCC1187 and HCC1143 cell lines and patient pleural effusions showed that Niclosamide inhibited Wnt/β-catenin pathway activation, down regulated LRP6, and decreased downstream β-catenin signaling.
- MCF10 non-malignant cell line did not respond to treatment with Niclosamide.
- The combination of TRA-8 and Niclosamide showed additive to synergistic secondary tumorsphere inhibition and further reduced Wnt/β-catenin activity.
- *Ex vivo* studies using 2LMP and SUM159 cell lines showed the combination treatment with TRA-8 and Niclosamide produced greater inhibition of tumorigenicity.
- *In vivo* studies showed that intraperitoneal administration of Niclosamide in combination with TRA-8 produced increased growth suppression of established 2LMP orthotopic tumor xenografts.

Reportable outcomes:
Publications related to aims:

1. **Angelina I. Londoño-Joshi,** Rebecca C. Arend, Laura Aristizabal, Wenyan Lu, Rajeev S. Samant, Brandon J. Metge, Bertha Hidalgo, Andres Forero, Albert F. LoBuglio, Yonghe Li, and Donald J. Buchsbaum: Effect of niclosamide on basal-like breast cancers. *Mol Cancer Thereputics*. *Under revision.*

2. Rebecca C. Arend, **Angelina I. Londoño-Joshi**, Rajeev S. Samant, Yonghe Li, Michael Conner, MD, Charles N. Landen, J. Michael Straughn, MD, and Donald J. Buchsbaum: Wnt/ß-catenin pathway inhibition by niclosamide: a therapeutic target for ovarian cancer. *Clinical Cancer Res*. In preparation.

3. Lin C, Lu W, Zhang W, **Londoño-Joshi AI**, Buchsbaum DJ, Bu G, Li Y: The C-terminal region Mesd peptide mimics full-length Mesd and acts as an inhibitor of Wnt/β-catenin signaling in cancer cells. *PLoS ONE* In press 2013.

4. **Londoño-Joshi AI**, Oliver PG, Li Y, Lee CH, Forero-Torres A, LoBuglio AF, Buchsbaum DJ: Basal-like breast cancer stem cells are sensitive to anti-DR5 mediated cytotoxicity. *Breast Cancer Res Treat* 133:437-445, 2012.

Publications not related to aims:

1. Janelle M. Fauci, **Angelina I. Londoño-Joshi**, J. Michael Straughn, Jr., MD,[a] Charles N. Landen, Soldano Ferrone, Donald J. Buchsbaum: Monoclonal antibody-based immunotherapy of ovarian cancer: targeting ovarian cancer cells with the B7-H3-specific mAb 376.96. *In preparation*

2. Swindall AF, **Londoño-Joshi AI**, Schultz MJ, Fineberg N, Buchsbaum DJ, Bellis SL: ST6Gal-I protein expression is upregulated in human epithelial tumors and correlates with stem cell markers in normal tissues and colon cancer cell lines. *Cancer Res* In press 2013.

3. Whitworth JM, **Londoño-Joshi AI**, Sellers JC, Oliver PG, Muccio DD, Atigadda VR, Straughn JM Jr, Buchsbaum DJ: The impact of novel retinoids in combination with platinum chemotherapy on ovarian cancer stem cells. *Gynecol Oncol* 125:226-230, 2012.

4. Bevis KS, McNally LR, Sellers JC, Della Manna D, **Londoño-Joshi AI**, Amm H, Straughn JM Jr, Buchsbaum DJ: Anti-tumor activity of an anti-DR5 monoclonal antibody, TRA-8, in combination with taxane/platinum-based chemotherapy in an ovarian cancer model. *Gynecol Oncol*, 121:193-199, 2011.

Awards:
1. AACR Minority Scholar in Cancer Research Award- 2013
2. Susan G. Komen for the Cure Travel Scholarship - AACR Annual Conference, Chicago, 2012.

Other:
1. Selected to serve on the Metastasis Research Society Council, International, 2013.
2. Attended the 3rd International Conference on Stem Cells and Cancer, New Delhi, India 2012.
3. AACR representative at One Voice Against Cancer Hill Day, Washington DC, 2012.

4. Attended annual meeting of the AACR, Chicago, IL 2012.
5. Please see additional reportable outcomes in *Curriculum Vitae* attached as Appendix A.

Conclusion:

Results described in this report indicate that targeting β-catenin in BLBC might be an effective means of increasing TRA-8 sensitivity. Combining TRA-8 with Niclosamide is an approach to ameliorate TRA-8 resistance in cancer cells by antagonizing the β-catenin pathway. Niclosamide is a well-studied drug in humans and is relatively inexpensive compared to other chemotherapeutic agents. The combination of these two well-tolerated drugs can be quickly translated into clinical trials, potentially helping BLBC patients who have very few treatment options available to them. This study provides valuable pre-clinical research results that gives important insight into using these novel agents in clinical trials in patients with BLBC.

References:

1. Liedtke, C., A.-M. Gonzalez-Angulo, and L. Pusztai, *Definition of Triple-Negative Breast Cancer and Relationship to Basal-Like Molecular Subtype.* PPO Updates Prin. Prac. Onc, 2010. **24**(2): p. 1-6.
2. Nielsen, T.O., et al., *Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma.* Clin Cancer Res, 2004. **10**(16): p. 5367-74.
3. Lakhani, S.R., et al., *Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype.* Clin Cancer Res, 2005. **11**(14): p. 5175-80.
4. Rakha, E.A., J.S. Reis-Filho, and I.O. Ellis, *Basal-like breast cancer: a critical review.* J Clin Oncol, 2008. **26**(15): p. 2568-81.
5. Honeth, G., et al., *The CD44+/CD24- phenotype is enriched in basal-like breast tumors.* Breast Cancer Res, 2008. **10**(3): p. R53.
6. Dontu, G., *Breast cancer stem cell markers - the rocky road to clinical applications.* Breast Cancer Res, 2008. **10**(5): p. 110.
7. Fillmore, C.M. and C. Kuperwasser, *Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy.* Breast Cancer Res, 2008. **10**(2): p. R25.
8. Giatromanolaki, A., et al., *The CD44+/CD24- phenotype relates to 'triple-negative' state and unfavorable prognosis in breast cancer patients.* Med Oncol.
9. Charafe-Jauffret, E., et al., *Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature.* Cancer Research, 2009. **69**(4): p. 1302-13.
10. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3983-8.
11. Ginestier, C., et al., *ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome.* Cell Stem Cell, 2007. **1**(5): p. 555-567.
12. Dean, M., *ABC transporters, drug resistance, and cancer stem cells.* J Mammary Gland Biol Neoplasia, 2009. **14**(1): p. 3-9.
13. Dave, B. and J. Chang, *Treatment resistance in stem cells and breast cancer.* J Mammary Gland Biol Neoplasia, 2009. **14**(1): p. 79-82.
14. Chuthapisith, S., et al., *Breast cancer chemoresistance: emerging importance of cancer stem cells.* Surg Oncol. **19**(1): p. 27-32.
15. King, T.D., M.J. Suto, and Y. Li, *The Wnt/beta-catenin signaling pathway: a potential therapeutic target in the treatment of triple negative breast cancer.* J Cell Biochem, 2012. **113**(1): p. 13-8.
16. Liu, C.C., et al., *LRP6 overexpression defines a class of breast cancer subtype and is a target for therapy.* Proc Natl Acad Sci U S A, 2010. **107**(11): p. 5136-41.
17. DiMeo, T.A., et al., *A novel lung metastasis signature links Wnt signaling with cancer cell self-renewal and epithelial-mesenchymal transition in basal-like breast cancer.* Cancer Res, 2009. **69**(13): p. 5364-73.
18. Lindvall, C., et al., *The Wnt co-receptor Lrp6 is required for normal mouse mammary gland development.* PLoS One, 2009. **4**(6): p. e5813.
19. Lu, W., et al., *Niclosamide suppresses cancer cell growth by inducing Wnt co-receptor LRP6 degradation and inhibiting the Wnt/beta-catenin pathway.* PLoS One, 2011. **6**(12): p. e29290.

20. Chen, M., et al., *The anti-helminthic niclosamide inhibits Wnt/Frizzled1 signaling.* Biochemistry, 2009. **48**(43): p. 10267-74.
21. Osada, T., et al., *Antihelminth compound niclosamide downregulates Wnt signaling and elicits antitumor responses in tumors with activating APC mutations.* Cancer Res, 2011. **71**(12): p. 4172-82.
22. Tanowitz, H.B., L.M. Weiss, and M. Wittner, *Diagnosis and treatment of intestinal helminths. I. Common intestinal cestodes.* Gastroenterologist, 1993. **1**(4): p. 265-73.
23. Craig, P. and A. Ito, *Intestinal cestodes.* Curr Opin Infect Dis, 2007. **20**(5): p. 524-32.
24. Merschjohann, K. and D. Steverding, *In vitro trypanocidal activity of the anti-helminthic drug niclosamide.* Exp Parasitol, 2008. **118**(4): p. 637-40.
25. Sack, U., et al., *Novel effect of antihelminthic Niclosamide on S100A4-mediated metastatic progression in colon cancer.* J Natl Cancer Inst, 2011. **103**(13): p. 1018-36.
26. Yo, Y.T., et al., *Growth inhibition of ovarian tumor-initiating cells by niclosamide.* Mol Cancer Ther, 2012. **11**(8): p. 1703-12.
27. Jin, Y., et al., *Antineoplastic mechanisms of niclosamide in acute myelogenous leukemia stem cells: inactivation of the NF-kappaB pathway and generation of reactive oxygen species.* Cancer Res, 2010. **70**(6): p. 2516-27.
28. Senthivinayagam, S., et al., *Caspase-mediated cleavage of beta-catenin precedes drug-induced apoptosis in resistant cancer cells.* J Biol Chem, 2009. **284**(20): p. 13577-88.
29. Cantarella, G., et al., *TRAIL-related neurotoxicity implies interaction with the Wnt pathway in human neuronal cells in vitro.* J Neurochem, 2008. **105**(5): p. 1915-23.
30. Rahman, M., et al., *TRAIL induces apoptosis in triple-negative breast cancer cells with a mesenchymal phenotype.* Breast Cancer Res Treat, 2009. **113**(2): p. 217-30.
31. Higuchi, H., et al., *Cholestasis increases tumor necrosis factor-related apoptotis-inducing ligand (TRAIL)-R2/DR5 expression and sensitizes the liver to TRAIL-mediated cytotoxicity.* J Pharmacol Exp Ther, 2002. **303**(2): p. 461-7.
32. Ichikawa, K., et al., *Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity.* Nat. Med., 2001. **7**: p. 954-960.
33. Buchsbaum, D.J., et al., *Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or radiation therapy in a human breast cancer model.* Clin. Cancer Res., 2003. **9**: p. 3731-3741.
34. Oliver, P.G., et al., *Effect of anti-DR5 and chemotherapy on basal-like breast cancer.* Breast Cancer Res Treat, 2012. **133**(2): p. 417-26.
35. Morrison, B.J., J.C. Steel, and J.C. Morris, *Sphere culture of murine lung cancer cell lines are enriched with cancer initiating cells.* PLoS One, 2012. **7**(11): p. e49752.
36. Londono-Joshi, A.I., et al., *Basal-like breast cancer stem cells are sensitive to anti-DR5 mediated cytotoxicity.* Breast Cancer Res Treat, 2012. **133**(2): p. 437-45.
37. Leung, J.Y., et al., *Activation of AXIN2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signaling.* J Biol Chem, 2002. **277**(24): p. 21657-65.

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Study Abroad-Universidad Complutense, Madrid, Spain 2005
Trans-Atlantic Student Science Exchange Program

Study Abroad-University of California, Santa Barbara 2003
Extended Learning Wildland Studies in Melbourne, Australia

AWARDS

- Department of Defense Breast Cancer Research Program Predoctoral Fellowship 2011-present
- UAB Graduated School
  - Oral Scientific Presentation Award 2012, 2013
- UAB Comprehensive Cancer Center
  - John R. Durant Award for Excellence in Cancer Research-Graduate Student Category 2011
- UAB Department of Pathology
  - Betty Spencer Pritchett Award for Outstanding Cancer Research 2011
  - AACR-Pancreatic Cancer Special Conference, Lake Tahoe, NV, travel award 2012
- Susan G. Komen for the Cure Travel Scholarship
  - American Association for Cancer Research (AACR) annual conference Chicago, IL 2012
  - AACR annual conference Washington, DC 2010
- Howard Hughes Medical Institute (HHMI)
  - HHMI Med-Grad Predoctoral Fellowship 2007-present
  - Science Education Alliance Teaching Assistant Award 2011
  - Genomic Analysis Training Janelia Farm, VA, travel award 2010
  - Translational Medicine Symposium, HHMI Peer Cluster Meeting, travel award 2010
  - AACR Annual Conference- Denver, CO, travel award 2009
- McNair Scholars Graduate School Program 2004-2006
- Pathway Scholars Program Summer Research Internship 2004
- Undergraduate Research Scholar 2002-2004
- College of Agricultural Life Science Honors Program 2002-2005
- Exxon Mobil Mathematics Youth Award-Hispanic Heritage Awards Foundation 2002
- POSSE Foundation Four-Year Full Tuition Undergraduate Scholarship 2001-2006

RESEARCH EXPERIENCE

UAB School of Medicine Division of Radiation Biology 2009-present
Predoctoral work in the lab of Dr. Donald J. Buchsbaum
Treatment of Breast Cancer Stem Cells with TRA-8 anti-DR5 Monoclonal Antibody

UAB Department of Cellular Molecular Biology 2008
Spring Rotation with Dr. Christopher Klug
Development of a syngeneic luciferase-pancreatic cancer model system for in vivo visualization of tumor progression
Gene Expression Profiling to Identify Novel Cell-Surface Antigens on Malignant Pancreatic Ductal Epithelium

UAB Department of Pathology Division of Molecular and Cellular Pathology 2008
Winter Rotation with Dr. Selvarangan Ponnazhagan
Chromatin Insulators and their Potential Use in rAAV Transgene Expression

UAB Department Immunology and Molecular and Cellular Biology 2007
Fall Rotation with Dr. Zedenk Hel
Induction of Long-Term Immunity via Transplantation of Antigen- Expressing Hematopoietic Stem Cells

UAB- Gene Therapy Center 2007
Summer Rotation with Dr. David Curiel and Dr. Larisa Pereboeva
Overexpression of HOXB4 in Mesenchymal Stem Cells

University of Wisconsin-Madison, Department of Radiology and Human Oncology 2003-2006
Undergraduate research mentor, Dr. Jamey Weichert
Validation of Non-Invasive Tissue Distribution by MicroPET Scanning In Mice
PLD Expression in Breast Cancer Tumor Cells Versus Normal Cells
In vivo Cell Trafficking of Metastasis of Colon Cancer

University of Wisconsin-Madison, Department of Biochemistry 2002-2003
HHMI investigator Dr. Judith Kimble
The Role of F17A9.3 in Germ Line Development of C. Elegans

PUBLICATIONS

** Contributed equally*

1. **Londoño-Joshi AI**, Buchsbaum DJ: Inhibition of Wnt co-receptor LRP6 sensitizes basal-like breast cancer stem cells to anti-DR5 monoclonal antibody alone or in combination with chemotherapy. *In preparation*

2. Fauci JM*, **Londoño-Joshi AI***, Sellers J, Zinn KR, Straughn MJ, Ferrone S, Buchsbaum DJ: Monoclonal antibody-based immunotherapy of ovarian cancer: targeting ovarian cancer cells with the B7-H3-specific mAb 376.96. *In preparation*

3. Swindall AF, **Londoño-Joshi AI**, Schultz MJ, Fineberg N, Buchsbaum DJ, Bellis SL: ST6Gal-I protein expression is highly upregulated in human epithelial tumors and correlates with stem cell markers in normal tissues and colon cancer cell lines *accepted in Cancer Research January 2013*

4. Lin C, Lu W, **Londoño-Joshi AI**, Zhang W, Bu G, Li Y: The C-terminal Region Mesd Peptide Mimics Full-length Mesd and Acts as An Inhibitor of Wnt/β-catenin Signaling in Cancer Cells *accepted in PLOS February 2013*

5. Whitworth JM*, **Londoño-Joshi AI***, Sellers JC, Oliver PG, Muccio DD, Atigadda VR, Straughn JM, Jr, Buchsbaum DJ: The impact of novel retinoids in combination with platinum chemotherapy on ovarian cancer stem cells. *Gynecol Oncol* In press, 2012

6. **Londoño-Joshi AI**, Oliver PG, Li Y, Lee CH, Forero-Torres A, LoBuglio AF, Buchsbaum DJ: Basal-like breast cancer stem cells are sensitive to anti-DR5 mediated cytotoxicity. *Breast Cancer Res Treat, Breast Cancer Res Treat* 133:437-445, 2012

7. Bevis KS, McNally LR, Sellers JC, Della Manna D, **Londoño-Joshi AI**, Amm H, Straughn JM, Jr, Buchsbaum DJ: Anti-tumor activity of an anti-DR5 monoclonal antibody, TRA-8, in combination with taxane/platinum-based chemotherapy in an ovarian cancer model. *Gynecol Oncol*, 121:193-199, 2011

ORAL PRESENTATIONS

**Invited Speaker*

1. *Investigation of death receptor-5 mediated apoptosis breast cancer stem cells. Hematology and Oncology Seminar Series, Birmingham, AL, 2010

2. *Investigation of death receptor-5 mediated apoptosis in basal-like breast cancer stem cells. Science Hour, Department of Radiation Oncology, Birmingham, AL, 2010

3. *Sensitivity of basal-like breast cancer stem cells to death receptor-5 mediated apoptosis. Komen Breast Cancer Research Roundtable, Birmingham, AL, 2010

4. Treatment of triple negative metastatic breast cancer stem cells with TRA-8 anti-death receptor 5 monoclonal antibody. Graduate Student Research Day, Birmingham, AL, 2009

5. Treatment of triple negative metastatic breast cancer with TRA-8 anti death receptor 5 (DR5) monoclonal antibody. Pathology Seminar, Birmingham, AL 2009

6. Development of a luciferase-expressing pancreatic cancer cell line. Pathology Seminar, Birmingham AL, 2008

7. Chromatin insulators and their potential use in rAAV transgene expression. Pathology Seminar, Birmingham, AL, 2008

8. Induction of long-term immunity via transplantation of antigen- expressing hematopoietic stem cell. Pathology Seminar, Birmingham, AL, 2007

9. Validation of non invasive tissue distribution by MicroPET scanning in mice, Undergraduate Symposium, Madison, WI, 2006

10. Phospholipase-D expression in breast cancer tumor cells versus normal cells. Pathways Scholars Symposium, Madison, WI, The National McNair Scholars Symposium, Berkeley, CA, 2004

11. Evaluating metastasis characteristics of CT-26 Cell with florescence labeled NM404. The Undergraduate Symposium, Madison, WI, 2004

12. F17A9.3 role in C. Elegans germ line development. The Undergraduate Research Scholar presentations, Madison, WI, 2002

POSTER PRESENTATIONS

1. **Londoño-Joshi AI**, Forero-Torres A, Fineberg NS, Oliver PG, Zhou T, LoBuglio AF, Buchsbaum DJ: Basal-like breast cancer stem cells are sensitive to combination treatment with anti-DR5 monoclonal antibody and chemotherapy
    - *Center for Clinical and Translational Science*, Birmingham, AL, 2011
    - *Proc Comprehensive Cancer Center 2011 Annual Research Retreat*, Birmingham, AL, 2011
    - *Pathology Research Day*, Birmingham, AL, 2011

2. **Londoño-Joshi AI**, Monti DL: Reading, writing, and arithmetic in the UAB phage explorations lab. *Science Education Alliance*, Janelia Farm, VA, 2001

3. **Londoño-Joshi AI**, Forero-Torres A, Fineberg NS, Oliver PG, Zhou T, LoBuglio AF, Buchsbaum DJ: Sensitivity of breast cancer stem cells to TRA-8 anti-DR5 monoclonal antibody
    - *AACR National Conference*, Washington, DC, 2010
    - *HHMI Southern Regional Conference*, Chapel Hill, NC, 2010

4. **Londoño-Joshi AI**, Forero-Torres A, Fineberg NS, Oliver PG, Zhou T, LoBuglio AF, Buchsbaum DJ: Treatment of triple negative breast cancer stem cells with TRA-8 anti-DR5 monoclonal antibody and 4HPR. Molecular and Cellular Pathology Research Day, Birmingham, AL, 2009

5. **Londoño-Joshi AI**, Forero-Torres A, Fineberg NS, Oliver PG, Zhou T, LoBuglio AF, Buchsbaum DJ: Treatment of triple negative breast cancer stem cells with TRA-8 anti-DR5 monoclonal antibody. Stem Cell Symposium and Cancer Center Retreat, Birmingham, AL, 2009

6. **Orozco AI**, Klug K: Gene expression profiling to identify novel cell-surface antigens on malignant pancreatic ductal epithelium. Howard Hughes Med-to Grad Regional Conference, Birmingham, AL, 2008

ABSTRACTS: non-presenting author

---

1. **Londoño-Joshi AI**, Della Manna DL, Ravi S, Oliver PG, Gillespie Y, Buchsbaum DJ: Treatment of glioblastoma cells with TRA-8 and AT-406. *The 2012 Brain SPORE Roundtable Meeting*, AZ, 2012

2. Ravi S, **Londoño-Joshi AI**, Oliver PG, Gillespie Y, Buchsbaum DJ: Characterization and treatment of glioma stem-like cells with temozolomide and TRA-8 combination therapy. *Patho-biology and Molecular Medicine Symposium*, Birmingham, AL, 2011

3. Whitworth JM, **Londoño-Joshi AI**, Sellers JC, Oliver PG, Muccio DD, Atigadda VR, Straughn JM, Jr, Buchsbaum DJ: The impact of novel retinoids in combination with platinum chemotherapy on ovarian cancer stem cells. *Proc Comprehensive Cancer Center 2011 Annual Research Retreat*, Birmingham, AL, 2011

4. Aristizabal A, **Londoño-Joshi AI**, Forero-Torres A, LoBuglio AF, Buchsbaum DJ: Characterization of $CD44^{+}/CD24^{-}/ALDH^{+}$ Marker expression as a model for drug development against basal-like breast cancer tumor initiating cells. *Undergraduate Research Expo*, Birmingham, AL, 2011

5. Ahmed A, Cain G, Clark D, Duckworth M, Dunkerley C, Johnston C, Mentreddy A, Nance C, **Londoño-Joshi AI,** Monti DL: Characterization of novel mycobacteriophages isolated from Alabama environmental samples. *Undergraduate Research Expo*, Birmingham, AL, 2011

6. Alakija O, Blair P, Chang M, Layfield S, Lee P, Liu M, Patel M, Thomas S, **Londono-Joshi AI**, Monti DL: Genomic analysis of the novel B1 mycobacteriophage, OSmaximus. *Undergraduate Research Expo*, Birmingham, AL, 2011

7. Forero-Torres A, Oliver PG, **Londoño-Joshi AI**, Zhou T, LoBuglio AF, Buchsbaum DJ: Death receptor 5, a therapeutic target for triple negative breast cancer. *The 2010 Breast Cancer Symposium*, National Harbor, MD, 2010

8. Buchsbaum DJ, Oliver PG, **Londoño-Joshi AI**, Zhou T, LoBuglio AF, Forero-Torres A: Death receptor 5 (DR5), a therapeutic target for triple negative breast cancer (TNBC). *The 2010 Breast SPORE Roundtable Meeting*, National Harbor, MD, 2010

9. **Londoño-Joshi AI**, Oliver PG, LoBuglio AF, Buchsbaum DJ: Treatment of triple negative breast cancer stem cells with TRA-8 anti-DR5 monoclonal antibody. *Proc Comprehensive Cancer Center 2009 Annual Research Retreat*, Birmingham, AL, 2009

PATENT

- Targeting Cancer Stem Cells March 2010. US Provisional Patent Application (# 61/315,413)

PUBLISHED GENOME SEQUENCES

- Mycobacterium phage OSmaximus, complete genome. GenBank: JN006064.1
- Mycobacterium phage Wee, complete genome. GenBank: HQ728524.1

TEACHING AND MENTORING EXPERIENCE

HHMI Science Education Alliance Phage Exploration, National Genomics Research Initiative Professor Dr. Denise Monti

- University of Alabama at Birmingham BY213 Phage Genomics I
  Teaching Assistant
  Fall 2010, Fall 2011, 4 credits lectured and taught lab for 2 months while professor was on leave
  16 students (primarily honors biology majors)

- University of Alabama at Birmingham BY214 Phage Genomics II
  Teaching Assistant
  Spring 2011, 4 credits lecture and lab
  16 students (primarily honors biology majors)

University of Alabama at Birmingham McNair Scholars Program

- Summer Research Peer Mentor
  Summer 2009, 2010 and 2011
  5 students (McNair Scholars undergraduate students)

- Tutor Undergraduate Students
  Fall 2009 Molecular Genetics, Spring 2010 Cancer Biology, and Fall 2010 Genetics

Research Mentoring at University of Alabama at Birmingham

- Supervised Undergraduate Student Research
  Summer 2009, 2010, 2011
  Andres Aristizabal; 2011 third place winner at state-wide poster competition
  Laura Aristizabal; 2012 second place winner at Summer Expo poster competition

- Supervised Graduate Student Rotation
  Saranya Ravi, Fall 2011; *Amber Guidry*, Spring 2010

- Supervised Gyn-Oncology Medical Fellow Research
  Keri Bevis, 2010; *Jenny Whitworth*, 2011; *Janelle Fauci*, 2012; *Rebecca Arend*, 2013

LEADERSHIP EXPERIENCE

Metastasis Research Society, USA representative on the Young Investigators Council 2013
Associate Member Council for the American Association for Cancer Research (AACR)
- Scientific Presentations Professional Advancement Series 2012-2013
- Programming, Advocacy Professional Advancement Series 2011-2012
- Sub-committee, Associate Membership and Recruitment 2011-2012

Toastmasters International Club
- President 2011-2012
- Vice-President of Education 2010-2011
- Founding Officer 2010

Graduate Student Association
- Treasurer 2010
- Budget Committee Chair 2010
- Cultural Activities Committee Co-Chair 2008-2010
- Senator- Pathology Department 2008-2011

Howard Hughes Med to Grad
- Peer Cluster Regional Southern Conference Coordinator 2008
- Volunteering Co-Chair 2008

La Colectiva (Hispanic Student Organization), President 2003-2006
Coalition of South American Students, Co-Chair 2003-2006
International Student Services Committee- Board member 2002-2006

EXTRACURRICULAR ACTIVITIES

- University of Alabama at Birmingham Graduate Biomedical Sciences
  - Cancer Biology Admission Committee 2010-2012
- Pancreatic Action Network Birmingham Affiliate
  - Helped organized first annual Purple Stride 5K, 1K Race, Birmingham, AL 2011-2013
  - Organized lab tour for pancreatic cancer patients and advocates, Birmingham, AL 2011
  - Represented Alabama, Jefferson County at Advocacy Days, Washington, DC 2011
- Hispanic Interest Coalition of Alabama: Medical translator, Birmingham AL 2008-present
- McNair Oral and Poster Presentation for Undergraduate Students: Judge, Birmingham, AL 2008-2011
- POSSE Foundation Undergraduate Student Scholarship: Selection committee, Chicago, IL 2006
- Bi-monthly weekend outings with handicapped and mentally disabled children, Madrid, Spain 2005
- Assisted terminally ill patients at Clinico Moncloa, Madrid Spain 2005
- American Multicultural Student Leadership Conference, Madison, WI 2004
- University of Wisconsin Hospital Burn Unit: Medical translator, Madison, WI 2004
- Boys and Girls Club: Holiday workshops and mentoring, Madison, WI 2003-2006
- World Health Day: Medical translator, Madison, WI 2003-2006
- Extended Learning Services, Little River Earth Sanctuary Environmental Project, Melbourne, Australia 2003

WORK EXPERIENCE

Hyde Park Dermatology- Medical Assistant/Insurance Coder 2007
Latin Solutions Marketing- Brand Ambassador 2006-2007
University of Wisconsin Health Service- Student Health Advocate 2003-2004
Southport Health Center- Assistant Manager 2000-2002
Instituto Cervantes of Chicago- Library Assistant 2001-2002
Illinois Public Interest Research Group- Canvasser 2001

CERTIFICATES

Toastmasters International Speaking and Leadership Certificates: Competent Communicator and Competent Leadership
Small Rodent Procedures: Pancreatic cancer orthotopic surgery. Mammary-fat-pad orthotopic injections. Intravenous, Intracardiac and Intraperitoneal injections
Health Insurance Probability and Accountability Act (HIPAA)
PADI Open Underwater Scuba Diver

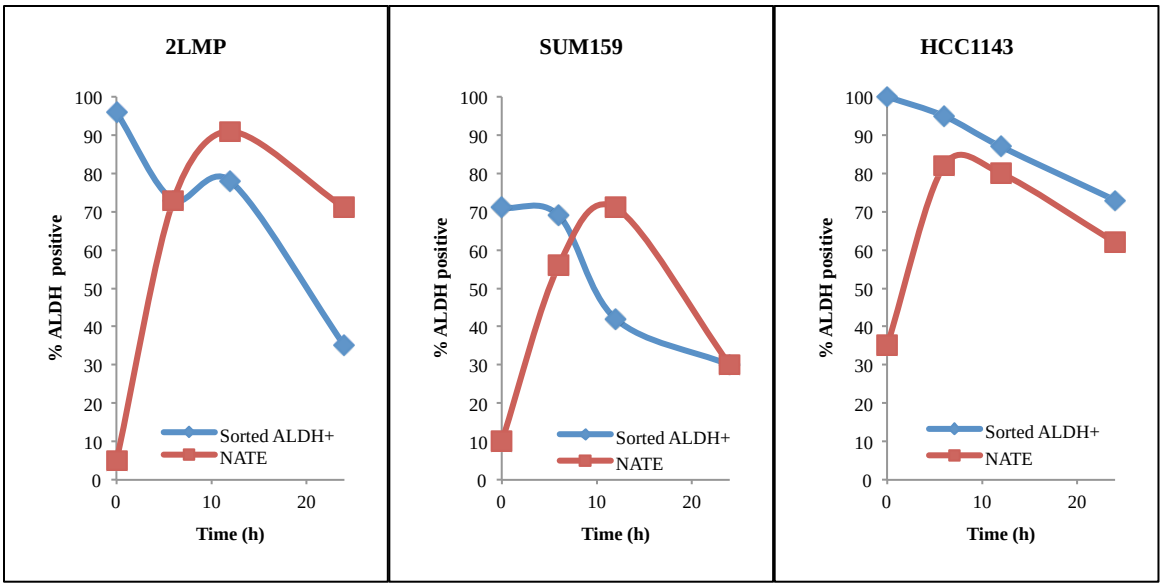
PROFESSIONAL MEMBERSHIPS

Society for Advancement of Chicanos and Native Americans in Science (SACNAS)
Center for Clinical and Translational Science (CCTS)
American Association for Cancer Research (AACR)
Birmingham International Center (BIC)
Metastasis Research Society (MRS)
Toastmasters International (TMI)

LANGUAGES

Native proficiency in English and Spanish

A.



B.

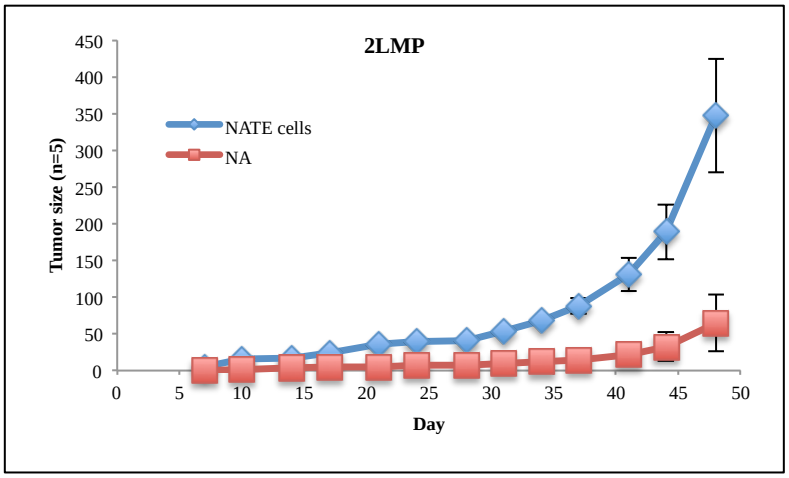


Figure 1. Characterization of non–adherent tumorsphere enriched (NATE) cells. (**A**) 2LMP, SUM159 and HCC1187 cells were FACS sorted for ALDH$^+$ cells and analyzed for ALDH expression at 6, 12 and 24 h after sorting (blue line) and compared to cells allowed to form tumorspheres (red line). NATE cells rapidly gained ALDH enzymatic expression reaching ~80% enrichment between 12-16 h. Cells harvested at this 12-16 h time point are called NATE cells. (**B**) 20,000 2LMP NATE cells were injected into the MFP of NOD/SCID mice and showed enhanced tumorigenicity compared to cells cultured in non-adherent conditions for 4 days (p= 0.01).

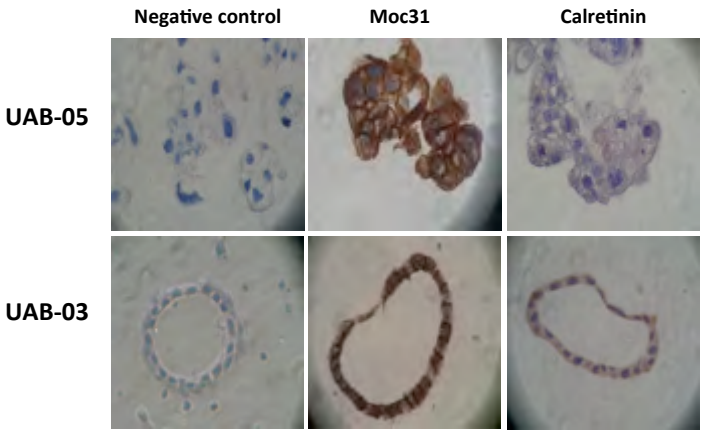


Figure 2. Immunohistochemistry characterization of patient pleural effusion samples. Patient sample name: UAB-05 and UAB-03 were stained for markers that indicate sample represents breast cancer cells and not mesothelial cells. 1st column negative control, 2nd column positive for Moc31 (adenocarcinoma), 3rd column negative for Calretinin (not mesothelial cells).

Cell Line	NATE IC_{50} uM	Adherent IC_{50} uM	*P* value NATE vs. Adherent
SUM159	0.11 (± 0.06)	0.31 (± 0.02)	0.015
HCC1187	0.18 (± 0.06)	1.0 (± 0.02)	0.0004
HCC1143	0.18 (± 0.08)	1.0 (± 0.02)	0.0002
2LMP	0.35 (± 0.13)	0.54 (± 0.34)	0.42

Table 1. Sensitivity of BLBC cell lines to niclosamide mediated cytotoxicity. SUM159, HCC1187, HCC1143 and 2LMP BLBC cell lines were treated for 48 h with niclosamide and analyzed for cell viability using ATPlite assay. Three of the four BLBC NATE cell lines showed significantly enhanced sensitivity compared to adherent cells. All individual experiments were assayed in quadruplicate and values represent the mean and SD from a minimum of three independent experiments.

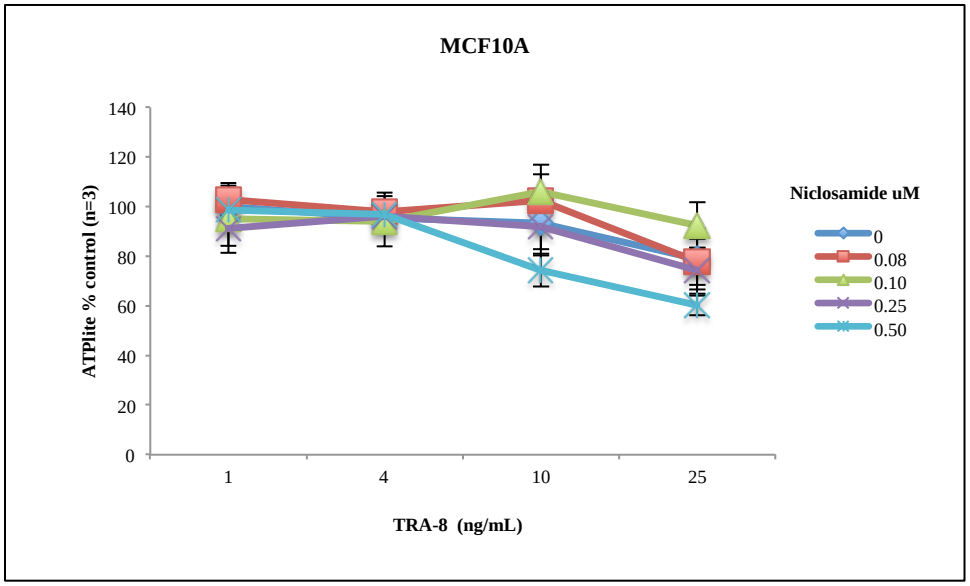


Figure 3. Sensitivity of MF10A cell line to niclosamide mediated cytotoxicity. Attached MCF10A cell line was treated for 48 h with Niclosamide and TRA-8 and analyzed for cell viability using ATPlite assay. All individual experiments were assayed in quadruplicate and values represent the mean and SE from three independent experiments.

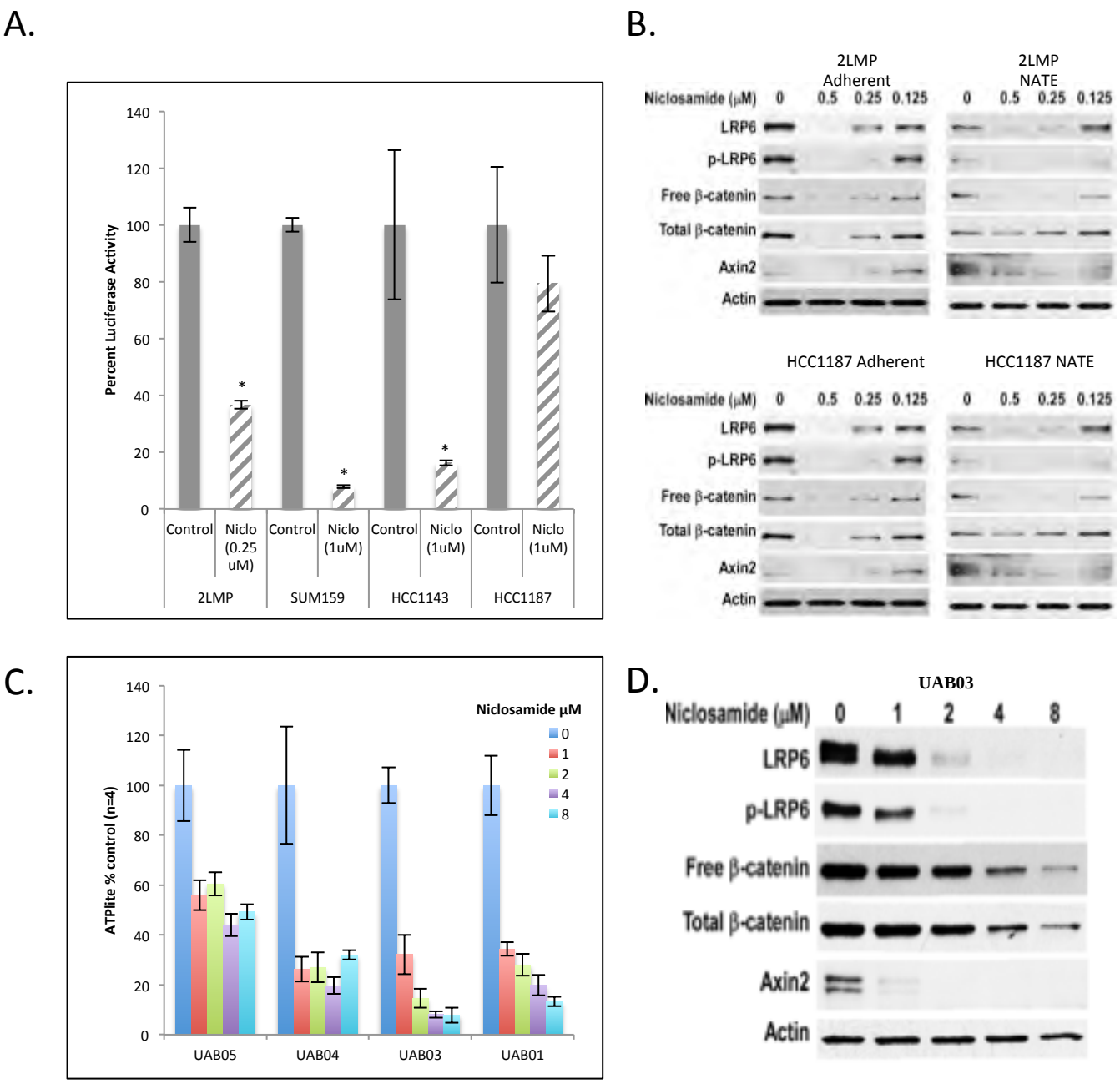


Figure 4. Niclosamide inhibits Wnt/β-catenin signaling in BLBC cell lines and patent samples. **(A)** 2LMP, SUM159, HCC143 and HCC1187 cells were treated Niclosamide for 24 h (SUM159, HCC1143 and HCC1187 were treated with 1 μM Niclosamide, and 2LMP with 0.25 μM Niclosamide). Niclosamide significantly inhibited activity of TCF/LEF luciferase TOPflash reporter in 2LMP, SUM159 and HCC1143 cell lines (*p= 0.0005, 0.002, 0.006). The experiment was performed in triplicate. The bars represent means ± SE. (**B)** Western blot analysis for 24 h treatment of 2LMP and HCC1187 cell line with niclosamide (0, 0.5, 0.25, 0.125 μM) on both adherent and NATE cells. LRP6 (top blot) p-LRP6 (second blot), free β-catenin (third blot), total β-catenin (fourth blot), Axin2 (fifth blot), Actin (sixth blot). Increasing doses of niclosamide reduced expression of Wnt/β-catenin signaling proteins (**C**) All four patient pleural effusion samples showed niclosamide mediated cytotoxicity with two samples showing a dose response (UAB03, UAB01 (1, 2, 4 and 8 μM). Samples were run in quadruplicates, bars represent means ± SE. (**D)** Western blot analysis for 24 h treatment of patient pleural effusion effusion sample UAB 03.

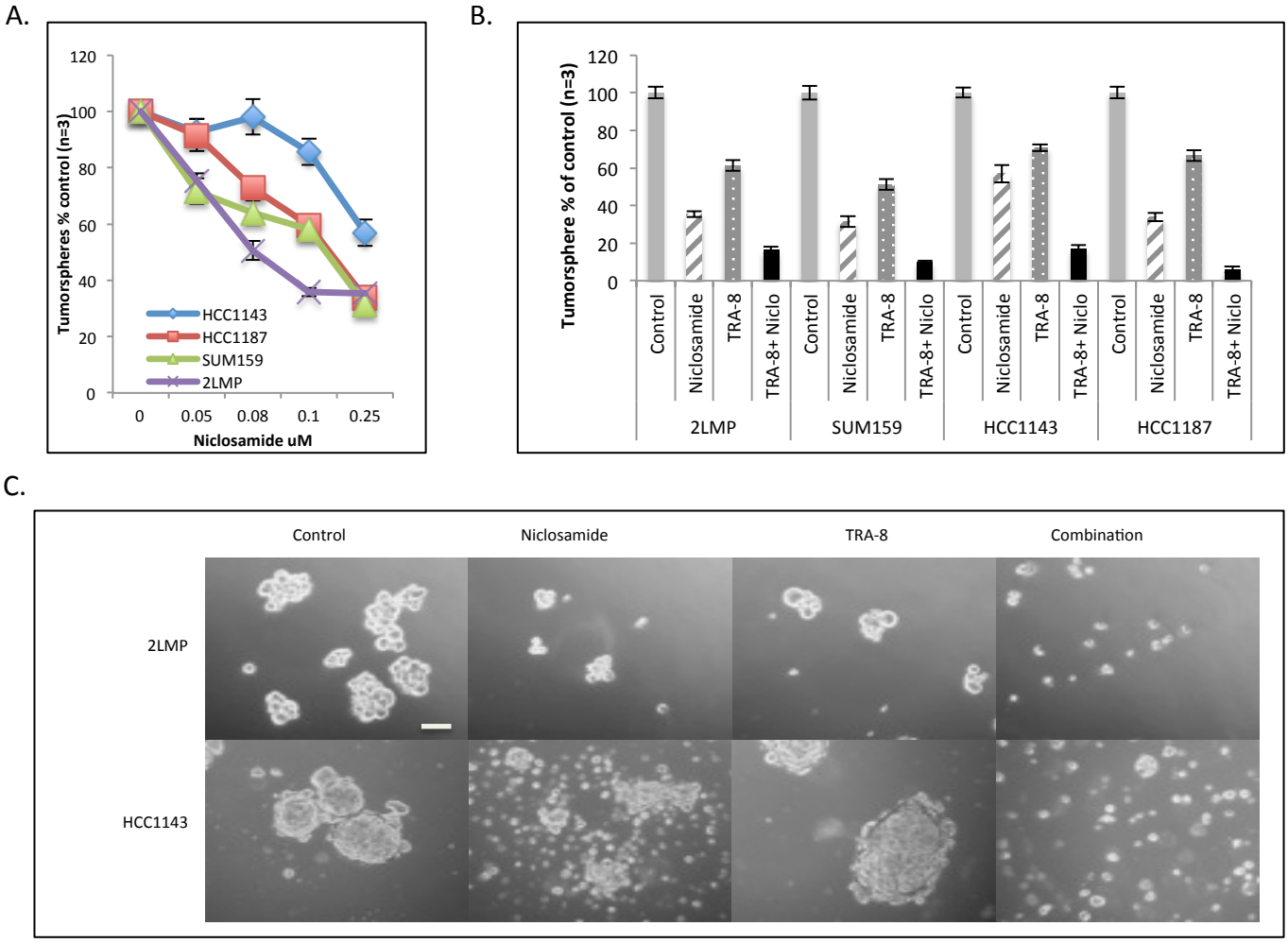


Figure 5. TRA-8 in combination with Niclosamide inhibits Wnt signaling in BLBC NATE cells. (**A**) Secondary tumorsphere inhibition by Niclosamide. HCC1143, HCC1187, SUM159 and 2LMP NATE cell lines were treated for 48 h with Niclosamide (0.05, 0.08, 0.1 and 0.25 µM). (**B**) Inhibition of secondary tumorsphere formation in combination with niclosamide and TRA-8. NATE cells were pre-treated with niclosamide for 24 (0.25 µM), followed with treatment with TRA-8 for an additional 24 h (1 ng/mL 2LMP, 0.5 ng/mL SUM159, 25 ng/mL HCC1143 and 5 ng/mL HCC1187). Secondary tumorsphere inhibition was visually counted using a reticle eye piece (50-150 µm). (**C**) Phase contrast pictures of 2LMP and HCC1143 cell lines pre-treated with niclosamide (0.25 µM) for 24 h, followed by TRA-8 (1 ng/mL 2LMP, 25 ng/mL HCC1143) and the combination, scale bar (100 µm). All individual experiments were assayed in quadruplicates, values represent the mean relative to control and error bars represent SE from three independent experiments. * $p < 0.007$

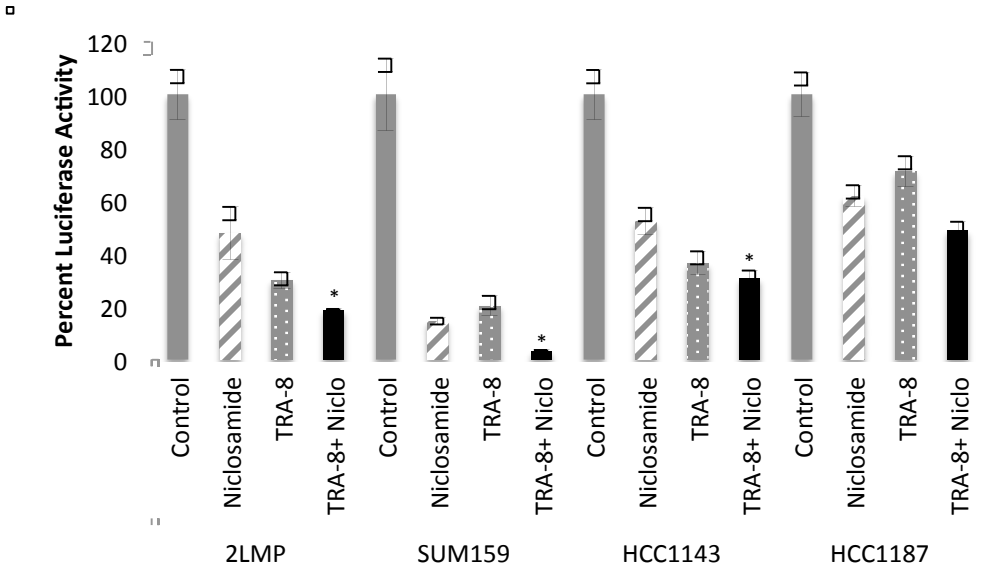


Figure 6. Niclosamide in combination with TRA-8 inhibits Wnt/β-catenin signaling. Activity of TCF/LEF reporter, TOPFlash was evaluated in 2LMP, SUM159, HCC1143 and HCC1187 adherent cells. All cell lines were treated with 0.25 μM niclosamide and 0.25 ng/mL TRA-8 for 24 h. The experiment was performed in triplicate. The error bars represent means ± SE.

	2LMP		SUM159	
	frequency	**Mean (SE)**	**frequency**	**Mean (SE)**
Control	5/5	189 mm^2	3/5	58 mm^2
TRA-8 (10nM)	3/5	158 mm^2	1/5	9 mm^2
Niclosamide (1uM)	4/5	127 mm^2	2/5	13 mm^2
TRA-8 + Niclosamide	4/5	71 mm^2	0/5	

Table 2. Effect of *ex vivo* treatment of BrCSC enriched cells on tumorigenicity in NOD/SCID mice. 2LMP and SUM159 tumorspheres were treated with TRA- 8, Niclosamide, combination and IgG control for 3 h and implanted into the MFP of groups of five NOD/SCID mice. The table represents the frequency of tumor engraftment and the average tumor size of the tumors that developed. In the 2LMP cell line 4/5 small, slow growing tumors were observed to develop within 44 days with TRA-8 and niclosamide treated cells while 5/5 tumors developed in the IgG, TRA-8 3/5, and Niclosamide treatment 4/5 groups. In the SUM159 cell line 3/5 tumors developed in the control group, 1/5 in TRA-8, 2/5 in the niclosamide treated group and 0/5 after 46 days after implantation.

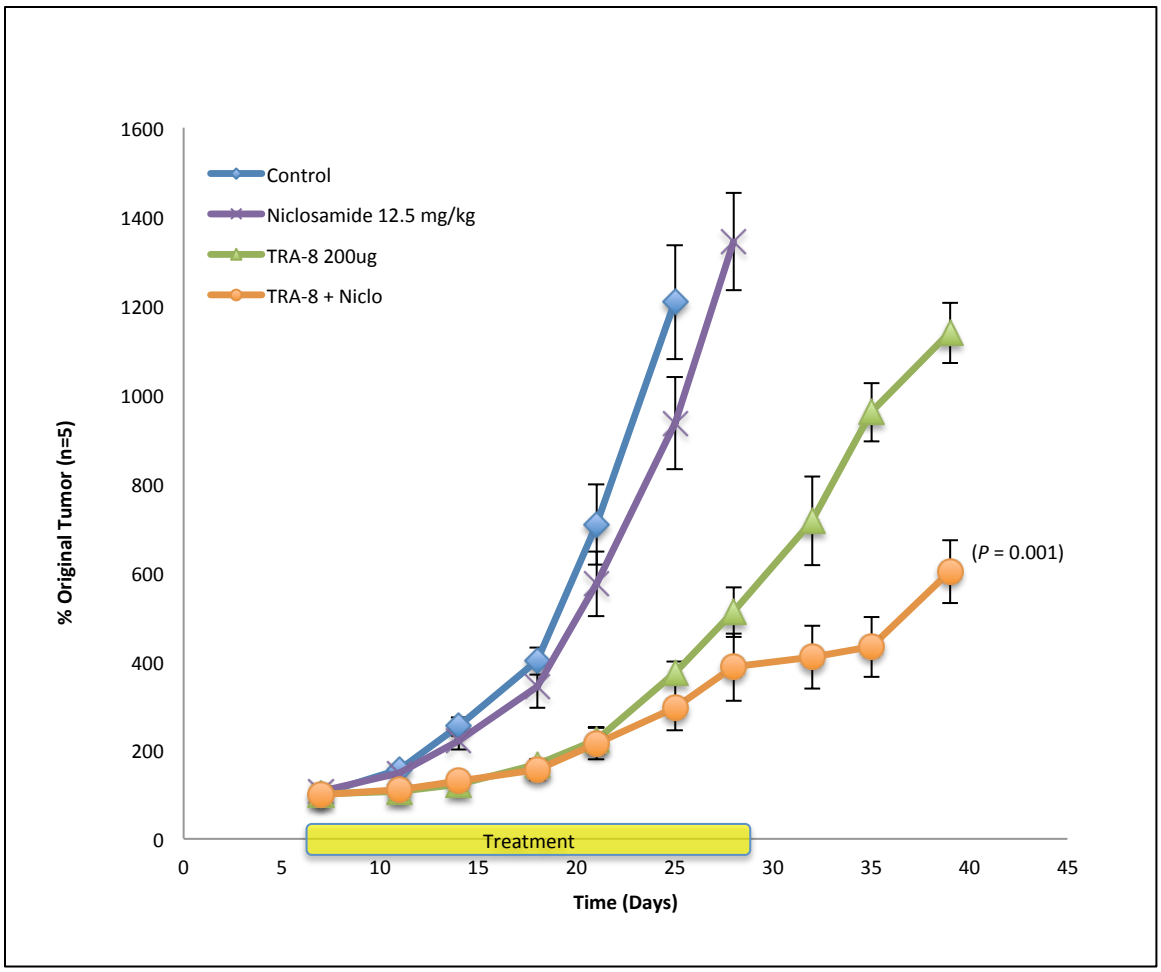


Figure 7. Antitumor effect of niclosamide in combination with TRA-8 *in vivo* on BLBC 2LMP cell line. Niclosamide in combination with TRA-8 reduced the tumor volume in athymic nude mice. Each point in the curve represents the mean + SE (n=5). The therapy started when tumors reached a volume of 16 mm^2. Niclosamide was given daily for 21 days, TRA-8 was given 2x weekly for 3 weeks. Niclosamide in combination with TRA-8 inhibited tumor growth. Tumor size was measured twice a week until mice were euthanized (combination vs. TRA-8 p=0.05, combination vs. control p=0.001).

Submitted
Molecular Cancer Therapeutics

TITLE:

Effect of niclosamide on basal-like breast cancers

AUTHORS:

Angelina I. Londoño-Joshi[1], Rebecca C. Arend[2], Laura Aristizabal[3], Wenyan Lu[4], Rajeev S. Samant[1], Brandon J. Metge[1], Bertha Hidalgo[5], Andres Forero[6], Albert F. LoBuglio[7], Yonghe Li[4], and Donald J. Buchsbaum[8]

INSTITUTIONS

Molecular and Cellular Pathology[1], Gynecologic Oncology[2], Biology[3], Southern Research Institute[4], Biostatistics, Section on Statistical Genetics[5], Hematology and Onocology[6], Medicine[7], Radiation Oncology[8], University of Alabama at Birmingham, Birmingham AL, USA.

Running Title: Niclosamide is cytotoxic to CSCs

Key words: TRA-8, Tigatuzumab, Death Receptor 5, Basal-like Breast Cancer, Cancer Stem Cells, Tumor Initiating Cells, LRP6, Wnt/β-catenin, Niclosamide (Niclocide).

CORRESPONDING AUTHORS:

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Donald J. Buchsbaum, Ph.D., Department of Radiation Oncology, University of Alabama at Birmingham, Birmingham, AL. Phone: (205) 934-7077. Fax: (205) 975-7060. E-mail: djb@uab.edu

Financial Support and Disclosure of Potential Conflicts of Interest: D.J. Buchsbaum, and A.F. LoBuglio: Intellectual property interest in TRA-8, Daiichi Sankyo.

ABSTRACT

Basal-like breast cancers (BLBCs) are poorly differentiated and display aggressive clinical behavior. These tumors become resistant to cytotoxic agents and tumor relapse has been attributed to the presence of cancer stem cells (CSCs). One of the pathways involved in CSC regulation is the Wnt/β-catenin signaling pathway. LRP6, a Wnt ligand receptor, is one of the critical elements of this pathway and could potentially be an excellent therapeutic target. Niclosamide has been shown to inhibit the Wnt/β-catenin signaling pathway by causing degradation of LRP6. TRA-8, a monoclonal antibody specific to TRAIL death receptor 5, is cytotoxic to BLBC cell lines and their CSC enriched populations. The goal of this study was to examine whether niclosamide is cytotoxic to BLBCs, specifically the CSC population, and if in combination with TRA-8 could produce increased cytotoxicity. Aldehyde dehydrogenase (ALDH) is a known marker of CSCs. By testing BLBC cells for ALDH expression by flow cytometry, we were able to isolate a non-adherent population of cells that have high ALDH expression. Niclosamide showed cytotoxicity against these non-adherent ALDH expressing cells in addition to adherent cells from four BLBC cell lines: 2LMP, SUM159, HCC1187 and HCC1143. Niclosamide produced reduced levels of LRP6, β -catenin and downstream Wnt/β-catenin signaling proteins in these cells. The combination of TRA-8 and niclosamide produced additive cytotoxicity and a reduction in Wnt/β-catenin activity. Niclosamide in combination with TRA-8 suppressed growth of 2LMP orthotopic tumor xenografts. These results suggest that niclosamide or congeners of this agent may be useful for the treatment of BLBC.

INTRODUCTION

Breast cancer is the second leading cause of cancer-related deaths in North American women. Basal-like breast cancer (BLBC), one of four subtypes of breast cancer, accounts for 13% of all breast carcinomas. While not as common as the other subtypes, BLBC is the most deadly and aggressive type (1, 2). This aggressiveness is due to the fact that it is also the most chemoresistant of the breast cancer subtypes (3). Biologically, it is characterized by a unique mRNA profile with CK5/6 expression and inactivation of *BRCA1*. Additionally, 70-90% of BLBC tumors are characterized as triple negative breast cancer (TNBC) because they lack progesterone and estrogen receptor expression and HER-2 amplification (1, 4, 5). The poor prognosis is linked to its enrichment for tumor initiating cells known as cancer stem cells (CSCs) (6-8). CSCs engage in self-renewal, induce tumors at low-cell density, and produce tumors with differentiated and heterogeneous cell profiles. Moreover, they exhibit gene expression profiles that diverge from their more differentiated cancer cell counterparts. Breast CSCs form tumorspheres *in vitro*, they are more tumorigenic in mice, and more resistant to standard chemotherapy and radiation than differentiated cells (9-11). In BLBC, CSCs are identified by their extracellular expression of CD44+/CD24- and elevated enzymatic activity of aldehyde dehydrogenase (ALDH) (12, 13). These CSCs are also identified based on the aberrant regulation of their self-renewing pathways, including Wnt, Hedgehog, and Notch signaling (12, 14).

One promising approach to prevent BLBC recurrence and metastasis is to target pathways that regulate CSCs such as the Wnt/β-catenin pathway (3, 15). The cell surface receptor LRP6, essential for Wnt/β-catenin signaling, is a potential target as its expression is up-regulated in 20-36% of human breast cancers and most significantly in the BLBC subtype. Suppression of LRP6 has been proven to be sufficient in inhibiting the Wnt signaling pathway in breast cancer; therefore, it is an excellent potential target for the treatment of BLBC (15-18).

Wnt proteins activate the Wnt pathway by binding to its surface receptor LRP5/6. This binding induces the receptor to interact with the transmembrane receptor, Frizzled (Fz), which leads to the subsequent phosphorylation of LRP5/6 (19). This leads to a build-up of β-catenin, an intracellular signal

transducer, in the cytoplasm. β-catenin can then translocate to the nucleus, where it interacts with T-cell transcription factor (TCF). This interaction triggers the transcription of the Wnt pathway target genes, which include survivin, Axin2 and Cyclin D1. The expression of these genes leads the cell to undergo proliferation, self-renewal and survival. In the absence of a Wnt ligand, β-catenin is tagged for degradation by the "destruction complex" comprised of adenomatous polyposis coli, Axin and GSK3β, thereby rendering the β-catenin target genes transcriptionally inactive.

The Wnt pathway can be inhibited at the extracellular level by secreted inhibitors such as DKKs or SFRPs (20, 21). Chemically this inhibition can be achieved by salinomycin, Mesd or niclosamide, which all are able to inhibit LRP6/LRP5 receptors (22-24). Wnt inhibitors, such as niclosamide, are reported to stimulate Fz internalization and promote LRP6 degradation, thus preventing proliferation and causing apoptosis (24-26). Niclosamide (trade name Niclocide) is a teniacide in the antihelminth family that has been FDA approved for the treatment of tapeworms. This safe, inexpensive drug has been used in humans for nearly 50 years (27-29). Niclosamide has also been shown to be cytotoxic against prostate and colorectal cancer, myelogenous leukemia, and in ovarian cancer it suppresses CSCs and metastasis (26, 30-32).

Wnt/β-catenin signaling is also inhibited by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) specifically by promoting caspase 3 and 8 mediated cleavage of β-catenin (33, 34). TRAIL also preferentially induces apoptosis in BLBC (35). TRA-8 is an agonistic monoclonal antibody (mAb) to TRAIL death receptor 5 (DR5) (36, 37). We have previously shown that TRA-8 can kill both parental and CSCs from BLBC (38, 39). We hypothesized that the suppression of canonical Wnt activity by niclosamide may have cytotoxic potential alone and may sensitize BLBC stem cells to treatment with TRA-8. In this study, we explored whether BLBC cell lines, non-adherent ALDH enriched (NAAE) cells, and cells isolated from BLBC patient pleural effusion samples were sensitive to niclosamide alone or in combination with TRA-8.

MATERIALS AND METHODS

Drugs and Antibodies

Niclosamide was purchased from Sigma (St. Louis, MO). Niclosamide for *in vitro* use was dissolved in DMSO at a 4.8 mM concentration and stored at 4°C until further use. For animal studies, niclosamide was dissolved in DMSO until a homogeneous suspension was observed at which time Cremophor was added to make a final solution of 25% DMSO and 75% Cremophor. The liquid was slowly inverted to obtain a clear orange solution, which was stored at 4°C. Purified TRA-8 (IgG1) mAb was prepared at the University of Alabama at Birmingham, as described previously, and was provided by Dr. Tong Zhou (36). IgG1 and isotype-specific IgG1 control antibody were obtained from Southern Biotechnology Associates (Birmingham, AL). ALDEFLUOR kit including DEAB was obtained from StemCell Technologies (Durham, NC). Monoclonal anti-phosphorylated-LRP6, anti-Axin2 and Cyclin D1 were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Monoclonal anti-β-catenin was purchased from BD Biosciences (San Jose, CA). Survivin antibody for Western blots was purchased from Santa Cruz (Santa Cruz, CA).

Cells, Cell Culture and Patient Pleural Effusion Samples

The 2LMP subclone of the human breast cancer cell line MDA-MB-231 was obtained from Dr. Marc Lippman (University of Miami, Miami, FL) and maintained in Improved-MEM supplemented with 10% FBS (Hyclone, Logan, UT). BLBC cell lines: HCC1187 and HCC1143 were obtained from American Type Culture Collection (Manassas, VA) and cultured according to supplier's directions. SUM159 was obtained from Asterand (Detroit, MI) and grown according to supplier's recommendation. MCF10A immortalized, non-transformed epithelial cells were obtained from American Type Culture Collection. All cell lines were maintained in antibiotic-free medium at 37°C in a 5% CO_2 atmosphere and routinely screened for mycoplasma contamination. With Institutional Review Board (IRB) approval, pleural effusion fluid was collected from advanced stage TNBC cancer patients after fluid required for diagnosis was sequestered. Cells were washed in phosphate buffered saline (PBS) (HyClone, Logan, UT)

and isolated via centrifugation at 400 rpm x 5 minutes. Cells were then plated in 96-well or 6-well ultra-low attachment plates (Corning, Corning, NY) and incubated in MEGM media (Lonza, Walkersville, MD) at 37°C atmosphere in 5% CO_2.

Generation and Characterization of Non-Adherent ALDH Enriched (NAAE) Cells

Adherent cells were harvested and seeded as single cells in 96-well ultra-low attachment plates (Corning, Corning, NY) at 40,000 cells/mL in mammary epithelial basal medium (MEBM) supplemented with bullet kit (bovine pituitary extract, insulin, human recombinant epidermal growth factor and hydrocortisone); complete media is called mammary epithelial cell growth medium (MEGM) (Lonza). These non-adherent cells were tested for ALDH activity at 6, 12, 48 and 72 hours after plating. This was compared to adherent tumor cells that were sorted for the ALDH+ population and subsequently analyzed for retention of ALDH expression at the same time points. The non-adherent cells were found to be enriched for ALDH at the 12-16 hour time-points, and therefore represent a CSC enriched population (Supplementary Fig. S1A). To verify enhanced tumorigenicity in these non-adherent ALDH enriched (NAAE) cells, they were injected into the mammary fat pad (MFP) of NOD/SCID mice and compared to non-adherent cells from the 72-hour time point that had reduced ALDH activity (Supplementary Fig. S1B). Tumors were measured twice weekly until mice were euthanized in accordance to IACUC regulations. The NAAE cells formed aggressive large tumors indicating their enrichment for CSCs compared to tumors formed by the non-adherent cells from the 72-hour time point (P = 0.01). NAAE cells were also analyzed by Western blot for LRP6, phosphorylated LRP6, free and total β-catenin (Supplementary Fig. S1C). The NAAE cells demonstrated higher free ß-catenin and lower LRP6 protein expression. Similar levels of phosphorylated LRP6 and total ß-catenin were observed in both NAAE and adherent populations. Elevated levels of free ß-catenin suggest that, this ALDH enriched population has elevated Wnt activity.

Cell Viability Assays using ATPlite

2LMP, SUM159, HCC1143 and HCC1187 cells were seeded at 2,000 cells/50 µL of media. Adherent cells were plated in optically clear tissue-cultured-treated 96-well black plates (Costar, Corning, NY) in their corresponding media. NAAE cells and patient sample cell preparations were plated as single cells, 2,000 cells/well, in 96-well low-attachment plates in MEGM media. Cells were treated with niclosamide (0.125-8 µM) for 48 hours. In combination treatment studies, after 24 hours exposure to niclosamide, TRA-8 (0.25-100 ng/mL) was added for an additional 24 hours before the cells were lysed and analyzed for viability using the ATPlite luminescence-based assay (PerkinElmer, Waltham, MA) as previously described (40). TRA-8 and niclosamide were diluted in culture medium immediately before use, and the final concentration of DMSO was always $\leq$ 0.001%. All samples were assayed in quadruplicate and are reported as the mean ± standard error (SE) from a minimum of three independent experiments.

TOPflash Luciferase Reporter Assay

Adherent cells were plated at 40,000 cells/well into 96-well adherent plates. Next day, the cells were transiently transfected with 0.05 mg of the TCF/LEF activity reporter (TOPflash) (plasmid from Dr. Randall Moon's laboratory, Upstate Biotechnology, Lake Placid, NY). NAAE cells and patient samples were assayed using Cignal Lenti Reporter TCF/LEF Reporter (Luc) (Qiagen, Germantown, MD). Cell lines were infected with lentiviral particles using an MOI of 10 and patient samples at an MOI of 25. Cells were transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM (Gibco/Invitrogen) as per the manufacturer's instructions. After 6 or 24 hours incubation (6 hours for plasmid, 24 hours for virus), cells were treated with niclosamide, TRA-8 or Wnt3A (R and D Systems, Minneapolis, MN). Total protein was harvested 24 hours post-treatment and luciferase activity was measured using a Turner 20/20 luminometer (Promega, Madison, WI). The luciferase reading was normalized to the total protein concentration as reported previously (41). The luciferase activity was normalized to untreated control and represented as a mean ± SE for a minimum of three replicates.

Western Blotting

2LMP and HCC1187 cell lines as NAAE and adherent cells, and patient samples were seeded ($1x10^6$ cells/well) in 6-well plates and treated for 24 hours with niclosamide (0, 0.5, 0.25, 0.125, 0.0625 μM) or for 2 hours with TRA-8 (25, 125 ng/mL). Cells were lysed in 0.5 ml of lysis buffer (PBS containing 1% Triton X-100 and 1 mM PMSF) at 4°C for 15 minutes. Equal quantities of protein were subjected to SDS-PAGE under reducing conditions. Following transfer to immobilon-P membrane, successive incubations with anti-phosphorylated-LRP6, anti-LRP6, anti-total β-catenin, anti-Axin2, anti-survivin, anti-cyclin D1 or anti-actin, and horseradish peroxidase-conjugated secondary antibody were carried out for 60-120 minutes at room temperature. The immunoreactive proteins were then detected using the ECL system (PerkinElmer). Films showing immunoreactive bands were scanned by Kodak Digital Science DC120 Zoom Digital Camera (Kodak, Rochester, NY). Cytosolic free β-catenin analysis was done with GST-E-cadherin binding assay (24). Uncomplexed cytosolic free β-catenin in 100 mg of total cell lysate was subjected to SDS-PAGE and detected using the mAb to β-catenin.

Immunohistochemistry

Patient pleural effusion samples were stained by immunohistochemistry for LRP6 (Cell Signaling Technology, Inc.), Moc31 and calretinin. Cells were harvested into histogel and imbibed into a paraffin block. For each sample, the staining intensity (0, 1+, 2+, 3+) and the percentage of cells staining positive (0-100%) were determined separately for the epithelial and stromal components. An H-score was calculated as the product of the intensity and the percentage of cells with positive staining. Cells collected from two BLBC patient pleural effusion samples (UAB03 and UAB05) were characterized by immunohistochemistry and both samples had an H-score of over 150 for Moc31 staining, and were negative for calretinin staining, which is a marker for mesothelial cells. This confirms that they are metastatic breast cancer epithelial cells rather than pleural mesothelial cells (data not shown). Patient samples also stained positive for LRP6 H-score 160 (UAB03), and 75 (UAB05) (Fig. 5C).

In Vitro Treatment of NAAE Cells

Tumorspheres were mechanically dissociated, plated in low attachment plates at 2,000 cells/well in 50 µL media, and allowed to form NAAE cells for 12-16 hours. Cells were pre-treated with niclosamide for 24 hours, followed by treatment with TRA-8 for an additional 24 hours. Two independent investigators used a reticle eyepiece to visually count tumorspheres. Mean tumorsphere inhibition was calculated relative to no treatment. Samples were run in quadruplicate in three independent experiments and error bars represent SE.

Generation of LRP6 Knockdown Cell Line

LRP6 protein expression was knocked down (KD) in the adherent 2LMP cell line using Mission Lentiviral transduction particles SHCLNV, MOI 1 of the TRCN0000033405 (Lot 01091309MN, $1.5x10^7$ TU/mL). Non-mammalian shRNA control Mission transduction particles SHC002V, MOI 3 (Lot 04301208MN, $2.6x10^7$ TU/mL) (Sigma-Aldrich, St. Louis, MO). Cells were selected using ready-made solution puromycin dihydrochloride (Sigma-Aldrich). LRP6 KD was confirmed using Western blot analysis of LRP6.

In Vivo Animal Study

Female athymic nude mice at 4 weeks of age were purchased from Harlan (Indianapolis, IN). *In vivo* generation of tumors was accomplished by resuspending $2x10^6$ 2LMP cells in 100 µL (1:1 Matrigel and PBS) and injecting cells into the mammary fat pad (MFP) of athymic nude mice (n=5). Mice were randomly assigned into groups and treated 7 days after tumor cell implantation when the tumors reached ~16 mm^2. Mice were intraperitoneally injected twice weekly with TRA-8 (200 µg) and niclosamide daily (30 mg/kg; 50% cremophore EL:50% DMSO) for 3 weeks. Tumor size (surface area) was calculated by $a \times b$, where a is the largest diameter and b is the diameter perpendicular to a. Tumors were measured with a Vernier caliper 2 times a week until mice were euthanized in accordance with the IACUC regulations.

Statistical Analysis

T-tests were used to compare means when appropriate. General linear models were used to make comparisons over time. Enhanced combination effect was further confirmed by a concentration–effect curve using the nonlinear regression method and isobologram methods (data not shown) (20). Experimental animal treatment groups were composed of 5 animals each to provide evidence of substantial tumor sensitivity to TRA-8 therapy with or without niclosamide. For xenograft models, tumor-doubling time (TDT) was estimated for each animal using empirical distribution, and median TDT between treatment groups was compared using Kruskal-Wallis nonparametric statistical test.

RESULTS

Niclosamide Induces Cytotoxicity to BLBC Cell Lines

We examined the cytotoxicity of niclosamide against BLBC tumor cell lines both as adherent cell populations and their NAAE components. As can be seen in Table 1, all four BLBC adherent cell lines were sensitive to niclosamide with an IC_{50} range of 0.33 - 1.9 μM drug concentrations. NAAE cell populations from SUM159, HCC1187 and HCC1143 BLBC cell lines were significantly more sensitive to niclosamide than the adherent cell populations with IC_{50} values of 0.17 - 0.18 μM (SUM159 $P < 0.05$, HCC1187 $P < 0.05$, HCC1143 $P < 0.0005$). The MCF10A mammary epithelial cells were not sensitive to niclosamide-mediated cytotoxicity (data not shown).

Niclosamide Inhibits Wnt/β-catenin Signaling in BLBC Cell Lines

To characterize the effect of niclosamide on the Wnt/β-catenin pathway, we performed the signaling TOPflash luciferase reporter assay on NAAE cells (Fig. 1A) and adherent cell lines (Supplementary Fig. S2). NAAE cells from all four BLBC cell lines showed significant inhibition of Wnt/B-catenin signaling following 24 hour incubation with niclosamide ($P < 0.01$) (Fig. 1A). In the presence of Wnt3A ligand, all four adherent cell lines were significantly inhibited by niclosamide ($P < 0.05$) while 3 of the 4 cell lines had niclosamide-mediated inhibition in the absence of the Wnt3A ligand ($P < 0.05$) (Supplementary Fig. S2). Thus, concentrations of niclosamide which induced BLBC cytotoxicity, inhibited the Wnt/β-catenin pathway.

Inhibition of the Wnt/β-catenin pathway was confirmed with Western blot analysis of Wnt/β-catenin signaling proteins after 24 hour treatment with niclosamide of adherent and NAAE cells (Fig. 1B). Niclosamide produced dose dependent reduction in the levels of LRP6, pLRP6, total and free β-catenin in both NAAE and adherent 2LMP cells (Fig. 1B). There was also a dose dependent reduction in Axin2, survivin and Cyclin D1, which are the targets of β-catenin signaling.

Effect of Niclosamide and TRA-8 on NAAE Cell Lines

Given our prior observation of TRA-8 cytotoxicity to BLBC cells (39) and their CSC enriched cell populations (38), we were interested in the effect of combining TRA-8 and niclosamide on NAAE cells. As can be seen in Figure 2, both niclosamide and TRA-8 inhibited secondary tumorsphere formation in all four cell lines. Drug interaction was additive for all four cell lines ($P < 0.03$).

Effect of Niclosamide and TRA-8 on Wnt/β-catenin Signaling

As can be seen in Fig. 3A, activity of TCF/LEF plasmid reporter in the TOPflash assay was evaluated in 2LMP, SUM159, HCC1143 and HCC1187 adherent cells. TOPflash activity was attempted on NAAE cells, but they could not be sufficiently transfected. The combination treatment of niclosamide and TRA-8 resulted in significant inhibition compared to untreated control for all four cell lines ($P < 0.05$). Combination treatment was also significantly better than niclosamide treatment for 3 out of the 4 cell lines (2LMP, SUM159, HCC1143) ($P < 0.05$ treatment vs. control, $P < 0.05$ combination treatment vs. niclosamide or TRA-8). The inhibition of TCF/LEF reporter activity was unexpected. To explore this effect, we examined the effect of TRA-8 on β-catenin. As seen in Fig. 3B, TRA-8 treatment of 2LMP and HCC1187 adherent and NAAE cells inhibited total β-catenin levels with evidence of fragmentation of the protein. This may explain the Wnt/β-catenin pathway inhibition. We were also interested if niclosamide inhibition of LRP6 could alter TRA-8 function (cytotoxicity). Knock down of LRP6 produced reduced survivin levels (Supplementary Fig. S3) and enhancement of TRA-8 mediated cytotoxicity of 2LMP cells. This is similar to the inhibition of survivin levels produced by niclosamide (Fig. 1B).

Effect of Niclosamide and TRA-8 on *In Vivo* 2LMP Tumors

Given the impressive *in vitro* observations of niclosamide and TRA-8 with BLBC cell lines, we carried out a therapy study using an orthotopic BLBC animal model. As seen in Fig. 4, animals receiving TRA-8 had a significant inhibition of tumor growth ($P < 0.05$), as previously reported (39). Animals treated with niclosamide at 30 mg/kg daily dose for 21 days had similar tumor growth inhibition while

animals treated with the combination of drugs had significant tumor growth inhibition compared to untreated controls ($P < 0.01$), or either single agent ($P < 0.05$).

Patient BLBC Samples are Sensitive to Niclosamide Alone and in Combination with TRA-8

Given the interesting niclosamide and TRA-8 effects on BLBC tumor cell lines, we explored their effects on patient BLBC tumor cells. Pleural effusion metastatic tumor cells were obtained from treatment resistant patients where tumors were BLBC. We documented that these cells (UAB01, UAB03, UAB04 and UAB05) were LRP6 membrane positive (data not shown). Niclosamide produced tumor cell cytotoxicity in all four tumor cell populations (Fig. 5A). UAB03 and UAB05 had adequate cells available and demonstrated niclosamide Wnt/β-catenin pathway inhibition (Fig. 5B). Western blot of UAB03 demonstrated dramatic inhibition of Wnt/β-catenin pathway proteins by niclosamide similar to its effect on BLBC cell lines (Fig. 5C). Finally, the combination of niclosamide and TRA-8 demonstrated enhanced tumor cell cytotoxicity compared to single agent effects. Thus, patient BLBC tumor cells appear to have similar effects to those seen with BLBC tumor cell lines (Fig. 5D) although requiring higher drug levels of niclosamide. This may reflect their drug resistance history and metastatic nature.

DISCUSSION

Most cancer treatment regimens assume that all cancer cells have equal malignant potential and respond similarly to therapy. More recently, therapies are being designed to target both bulk tumor cells and CSCs with the goal to prevent recurrence and metastasis. Therefore, therapies that target both the CSC maintenance pathways and induce apoptosis in the bulk tumor cell population could potentially result in increased chances for survival. We have previously described that TRA-8 preferentially kills BLBC cell lines and xenografts and their CSC population. In an effort to improve on this strategy, this study demonstrated that niclosamide, a potent Wnt/β-catenin inhibitor, produced similar cytotoxicity with bulk tumor cells and CSC enriched cell populations.

Because CSCs represent a small fraction of the total tumor cell population, it is difficult to run mechanistic studies with these cells especially by techniques such as Western blot and the TOPflash reporter assay that require a large number of cells. Therefore, NAAE cells were generated as a tool to analyze the effect of drugs on CSC enriched populations. NAAE cells acquired elevated ALDH activity between 12-16 hours after plating in low attachment conditions, and subsequently returned to the pre-sorted population expression levels (Supplementary Fig. S1A). Our findings imply that sorting may not be necessary to enrich for CSC characteristics if treatments are conducted within a short time point after plating. Our results also indicate that treatments that are targeted toward CSCs should not be carried out on sorted populations after 48 hours from sorting due to depletion of initial enrichment for CSC marker expression. *In vivo* tumorigenicity studies were used to functionally validate NAAE cells. Tumor uptake and growth rate of NAAE xenografts were compared to tumors generated from non-adherent cells depleted of ALDH activity. NAAE xenografts developed earlier and formed larger tumors over time, indicating enrichment for tumor initiating CSCs (Supplementary Fig. S1B). Western blot analysis of the NAAE cells and adherent parental population, demonstrate that NAAE cells have up regulation of free ß-catenin. This observation further helps characterize NAAE cells as upregulating Wnt signaling, which is a key development pathway and it has been shown that its deregulation leads to oncogenesis. Future

studies will characterize NAAE cells and look at epithelial-mesenchymal transition and mesenchymal-epithelial transition markers that could potentially influence observed dynamic CSC marker expression (42).

The involvement of Wnt/β-catenin signaling in BLBC and CSCs has been well documented (15). High-throughput screening studies identified niclosamide as a potent Wnt pathway inhibitor (43). Nonetheless, like many inhibitors of signaling pathways, niclosamide also affects other pathways than Wnt, such as NF-κB, Notch, ROS, mTORC1, and Stat3 (44). However, multiple publications by various groups show that niclosamide is a potent Wnt inhibitor (24-26). Furthermore, niclosamide has been shown to be cytotoxic and target the Wnt/β-catenin signaling pathway in cell lines from several cancer models such colon (45), ovarian (32), osteosarcoma (25), myeloma (46), and breast cancer (24). We wanted to investigate niclosamide cytotoxicity in additional BLBC cell lines including 2LMP (a metastatic clone of MDA-MB-231), SUM159, HCC1187 and HCC1143, and patient pleural effusion samples from BLBC patients. Furthermore, CSC enriched NAAE cells were generated from these cell lines to test their sensitivity to niclosamide treatment. All four BLBC cell lines were sensitive to niclosamide at an $IC_{50} < 2$ μM, and the NAAE cells derived from these lines were even more sensitive to treatment with niclosamide (Table 1). The sensitivity of NAAE cells to niclosamide treatment further confirms the role of Wnt in the CSC enriched population. Furthermore, Western blot analysis of NAAE cells demonstrates that they have reduced levels of LRP6, thus their enhanced sensitivity is not due to overexpression of LRP6 on the NAAE cells but possibly due to their dependency on Wnt signaling, indicated by elevated levels of free ß-catenin. Moreover, there was significant cytotoxicity of niclosamide against BLBC cells isolated from patient pleural effusion samples that were resistant to chemotherapy (Fig. 5A). These findings are particularly exciting considering these patient samples were resistant to *in vitro* treatment with chemotherapy and Notch inhibitors (data not shown). Also *in vitro* cytotoxicity of these patient samples was within the range of blood concentrations (1.8 - 17 μM) achieved when healthy patients were given the 2 g dose of niclosamide for the treatment of tapeworms. There is little or no data on repeat dose schedules.

Functional inhibition of the Wnt/β-catenin signaling pathway was confirmed using the TOPflash luciferase reporter assay. Following 24-hour treatment with niclosamide, all four BLBC cell lines in adherent conditions showed significant inhibition of Wnt/β-catenin signaling (Fig. 1A). The TOPflash results were validated by investigating inhibition of Wnt/β-catenin using Western blot analysis of two BLBC cell lines (2LMP and HCC1187) (Fig. 1B). These studies clearly demonstrated that niclosamide depleted LRP6 protein as well as total and free β-catenin protein expression and its target proteins, survivin, Cyclin D1 and Axin2. The ability of niclosamide to deplete protein expression of survivin compared to untreated control cells may explain the increase in apoptotic sensitivity. Survivin normally inhibits caspase-9 activation within the apoptosome, a key step in the apoptotic cascade (47). The ability of niclosamide to inhibit survivin is a potential mechanism by which the addition of TRA-8 is resulting in *in vitro* and *in vivo* enhanced antitumor activity against BLBC cells. 2LMP LRP6 KD cells showed reduced survivin activity compared to shRNA control (Supplementary Fig. S3). We also demonstrated that these 2LMP LRP6 KD cells had enhanced sensitivity to TRA-8, thus further validating our hypothesis that inhibition of canonical Wnt signaling can sensitize cells to death receptor mediated apoptosis (Fig. 3).

Additionally, it appears that the extrinsic apoptotic pathway can regulate β-catenin activity. TRAIL has been shown to decrease full-length β-catenin expression. This relationship between β-catenin activity and TRAIL suggests that inhibiting β-catenin activity could promote TRA-8 sensitivity (34, 48). This study demonstrated a novel mechanism for modulation of β-catenin activity by TRA-8. These observations were made using both luciferase TOPflash reporter assay and Western blot analysis of cleaved ß-catenin (Figs. 3A and 3B). Future studies will focus on characterizing the mechanism by which TRA-8 can inhibit β-catenin activity and the results could help further elucidate how TRA-8 preferentially induces apoptosis in CSCs.

Overall, this study provides new insights into the treatment of CSCs from BLBC. It further supports existing literature that inhibition of Wnt signaling using niclosamide produces enhanced cytotoxicity against CSCs and the bulk population of cancer cells. Additionally, we demonstrated the

combination of niclosamide and TRA-8 can target the CSC subpopulation and potentially prevent recurrence and metastasis in patients with BLBC. The combination of these two agents or similar compounds can potentially be translated into clinical trials, helping BLBC patients who have very few treatment options available to them.

AUTHORS' CONTRIBUTION

All authors have read and approved the final manuscript. AILJ, RSS, AF, AFL, YL and DJB developed the idea. AILJ, LA, WL, BJM, and YL performed the experiments, analyzed the data, and prepared the manuscript. AILJ and DJB initially conceived the idea, and participated in the experimental design and manuscript preparation.

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REFERENCES

1. Liedtke C, Gonzalez-Angulo A-M, Pusztai L. Definition of triple-negative breast cancer and relationship to basal-like molecular subtype. PPO Updates: Prin Prac Oncol. 2010;24:1-6.

2. American Cancer Society. Cancer Facts and Figures 2013.

3. Rakha EA, Reis-Filho JS, Ellis IO. Basal-like breast cancer: a critical review. J Clin Oncol. 2008;26:2568-81.

4. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res. 2004;10:5367-74.

5. Lakhani SR, Reis-Filho JS, Fulford L, Penault-Llorca F, van der Vijver M, Parry S, et al. Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype. Clin Cancer Res. 2005;11:5175-80.

6. Honeth G, Bendahl PO, Ringner M, Saal LH, Gruvberger-Saal SK, Lovgren K, et al. The CD44+/CD24- phenotype is enriched in basal-like breast tumors. Breast Cancer Res. 2008;10:R53.

7. Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, et al. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. Cancer Res. 2009;69:1302-13.

8. Tsang JY, Huang YH, Luo MH, Ni YB, Chan SK, Lui PC, et al. Cancer stem cell markers are associated with adverse biomarker profiles and molecular subtypes of breast cancer. Breast Cancer Res Treat. 2012;136:407-17.

9. Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. Breast Cancer Res. 2008;10:R25.

10. Goldthwaite J, C.A. . Are Stem Cells Involved in Cancer? Regenerative Medicine. [cited; 89-95]. Available from: </info/scireport/2006report.htm>

11. Grimshaw MJ, Cooper L, Papazisis K, Coleman JA, Bohnenkamp HR, Chiapero-Stanke L, et al. Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. Breast Cancer Res. 2008;10:R52.

12. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell. 2007;1:555-67.

13. Croker AK, Goodale D, Chu J, Postenka C, Hedley BD, Hess DA, et al. High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. J Cell Mol Med. 2009;13:2236-52.

14. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA. 2003;100:3983-8.

15. King TD, Suto MJ, Li Y. The Wnt/beta-catenin signaling pathway: a potential therapeutic target in the treatment of triple negative breast cancer. J Cell Biochem. 2012;113:13-8.

16. DiMeo TA, Anderson K, Phadke P, Fan C, Perou CM, Naber S, et al. A novel lung metastasis signature links Wnt signaling with cancer cell self-renewal and epithelial-mesenchymal transition in basal-like breast cancer. Cancer Res. 2009;69:5364-73.

17. Lindvall C, Zylstra CR, Evans N, West RA, Dykema K, Furge KA, et al. The Wnt co-receptor Lrp6 is required for normal mouse mammary gland development. PLoS ONE. 2009;4:e5813.

18. Liu CC, Prior J, Piwnica-Worms D, Bu G. LRP6 overexpression defines a class of breast cancer subtype and is a target for therapy. Proc Natl Acad Sci USA. 2010;107:5136-41.

19. Joiner DM, Ke J, Zhong Z, Xu HE, Williams BO. LRP5 and LRP6 in development and disease. Trends Endocrinol Metab. 2013;24:31-9.

20. Li Y, Lu W, King TD, Liu CC, Bijur GN, Bu G. Dkk1 stabilizes Wnt co-receptor LRP6: implication for Wnt ligand-induced LRP6 down-regulation. PLoS ONE. 2010;5:e11014.

21. Lavergne E, Hendaoui I, Coulouarn C, Ribault C, Leseur J, Eliat PA, et al. Blocking Wnt signaling by SFRP-like molecules inhibits in vivo cell proliferation and tumor growth in cells carrying active beta-catenin. Oncogene. 2011;30:423-33.

22. Lu W, Liu CC, Thottassery JV, Bu G, Li Y. Mesd is a universal inhibitor of Wnt coreceptors LRP5 and LRP6 and blocks Wnt/beta-catenin signaling in cancer cells. Biochemistry. 2010;49:4635-43.

23. Lu D, Choi MY, Yu J, Castro JE, Kipps TJ, Carson DA. Salinomycin inhibits Wnt signaling and selectively induces apoptosis in chronic lymphocytic leukemia cells. Proc Natl Acad Sci USA. 2011;108:13253-7.

24. Lu W, Lin C, Roberts MJ, Waud WR, Piazza GA, Li Y. Niclosamide suppresses cancer cell growth by inducing Wnt co-receptor LRP6 degradation and inhibiting the Wnt/beta-catenin pathway. PLoS ONE. 2011;6:e29290.

25. Chen M, Wang J, Lu J, Bond MC, Ren XR, Lyerly HK, et al. The anti-helminthic niclosamide inhibits Wnt/Frizzled1 signaling. Biochemistry. 2009;48:10267-74.

26. Osada T, Chen M, Yang XY, Spasojevic I, Vandeusen JB, Hsu D, et al. Antihelminth compound niclosamide downregulates Wnt signaling and elicits antitumor responses in tumors with activating APC mutations. Cancer Res. 2011;71:4172-82.

27. Tanowitz HB, Weiss LM, Wittner M. Diagnosis and treatment of intestinal helminths. I. Common intestinal cestodes. The Gastroenterologist. 1993;1:265-73.

28. Craig P, Ito A. Intestinal cestodes. Current opinion in infectious diseases. 2007;20:524-32.

29. Merschjohann K, Steverding D. In vitro trypanocidal activity of the anti-helminthic drug niclosamide. Experimental parasitology. 2008;118:637-40.

30. Jin Y, Lu Z, Ding K, Li J, Du X, Chen C, et al. Antineoplastic mechanisms of niclosamide in acute myelogenous leukemia stem cells: inactivation of the NF-kappaB pathway and generation of reactive oxygen species. Cancer Res. 2010;70:2516-27.

31. Sack U, Walther W, Scudiero D, Selby M, Kobelt D, Lemm M, et al. Novel effect of antihelminthic Niclosamide on S100A4-mediated metastatic progression in colon cancer. J Natl Cancer Inst. 2011;103:1018-36.

32. Yo YT, Lin YW, Wang YC, Balch C, Huang RL, Chan MW, et al. Growth inhibition of ovarian tumor-initiating cells by niclosamide. Mol Cancer Ther. 2012;11:1703-12.

33. Cantarella G, Di Benedetto G, Pezzino S, Risuglia N, Bernardini R. TRAIL-related neurotoxicity implies interaction with the Wnt pathway in human neuronal cells in vitro. J Neurochem. 2008;105:1915-23.

34. Senthivinayagam S, Mishra P, Paramasivam SK, Yallapragada S, Chatterjee M, Wong L, et al. Caspase-mediated cleavage of beta-catenin precedes drug-induced apoptosis in resistant cancer cells. J Biol Chem. 2009;284:13577-88.

35. Rahman M, Davis SR, Pumphrey JG, Bao J, Nau MM, Meltzer PS, et al. TRAIL induces apoptosis in triple-negative breast cancer cells with a mesenchymal phenotype. Breast Cancer Res Treat. 2009;113:217-30.

36. Ichikawa K, Liu W, Zhao L, Wang Z, Liu D, Ohtsuka T, et al. Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. Nat Med. 2001;7:954-60.

37. Buchsbaum DJ, Zhou T, Grizzle WE, Oliver PG, Hammond CJ, Zhang S, et al. Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or radiation therapy in a human breast cancer model. Clin Cancer Res. 2003;9:3731-41.

38. Londono-Joshi AI, Oliver PG, Li Y, Lee CH, Forero-Torres A, Lobuglio AF, et al. Basal-like breast cancer stem cells are sensitive to anti-DR5 mediated cytotoxicity. Breast Cancer Res Treat. 2012;133:437-45.

39. Oliver PG, Lobuglio AF, Zhou T, Forero A, Kim H, Zinn KR, et al. Effect of anti-DR5 and chemotherapy on basal-like breast cancer. Breast Cancer Res Treat. 2012;133:417-26.

40. Oliver PG, LoBuglio AF, Zinn KR, Kim H, Nan L, Zhou T, et al. Treatment of human colon cancer xenografts with TRA-8 anti-death receptor 5 antibody alone or in combination with CPT-11. Clin Cancer Res. 2008;14:2180-9.

41. Mitra A, Menezes ME, Shevde LA, Samant RS. DNAJB6 induces degradation of beta-catenin and causes partial reversal of mesenchymal phenotype. J Biol Chem. 2010;285:24686-94.

42. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;133:704-15.

43. Chen W, Chen M, Barak LS. Development of small molecules targeting the Wnt pathway for the treatment of colon cancer: a high-throughput screening approach. Am J Physiol Gastrointest Liver Physiol. 2010;299:G293-300.

44. Pan JX, Ding K, Wang CY. Niclosamide, an old antihelminthic agent, demonstrates antitumor activity by blocking multiple signaling pathways of cancer stem cells. Chinese journal of cancer. 2012;31:178-84.

45. Waaler J, Machon O, Tumova L, Dinh H, Korinek V, Wilson SR, et al. A novel tankyrase inhibitor decreases canonical Wnt signaling in colon carcinoma cells and reduces tumor growth in conditional APC mutant mice. Cancer Res. 2012;72:2822-32.

46. Khanim FL, Merrick BA, Giles HV, Jankute M, Jackson JB, Giles LJ, et al. Redeployment-based drug screening identifies the anti-helminthic niclosamide as anti-myeloma therapy that also reduces free light chain production. Blood cancer journal. 2011;1:e39.

47. Altieri DC. Molecular circuits of apoptosis regulation and cell division control: the survivin paradigm. J Cell Biochem. 2004;92:656-63.

48. Higuchi H, Bronk SF, Taniai M, Canbay A, Gores GJ. Cholestasis increases tumor necrosis factor-related apoptotis-inducing ligand (TRAIL)-R2/DR5 expression and sensitizes the liver to TRAIL-mediated cytotoxicity. J Pharmacol Exp Ther. 2002;303:461-7.

Table 1. Sensitivity of BLBC cell lines to niclosamide mediated cytotoxicity. SUM159, HCC1187, HCC1143 and 2LMP BLBC NAAE and adherent cell lines were treated with niclosamide for 48 hours and analyzed for viability using the ATPlite assay. Individual experiments were assayed in quadruplicate and values represent the mean and SD of three independent experiments. SUM159 $P < 0.05$, HCC1187 $P < 0.05$, HCC1143 $P < 0.0005$, 2LMP $P > 0.05$.

Cell Line	NAAE IC_{50} µM	Adherent IC_{50} µM
SUM159	0.18 (± 0.09)	0.33 (± 0.04)
HCC1187	0.18 (± 0.06)	1.9 (± 1.15)
HCC1143	0.17 (± 0.09)	1.1 (± 0.12)
2LMP	0.29 (± 0.19)	0.44 (± 0.29)

Figure Legends

Figure 1. Niclosamide effect on Wnt/β-catenin signaling. (**A**) Activity of TCF/LEF viral reporter in the TOPflash assay was evaluated in 2LMP, HCC1187, SUM159 and HCC1143 NAAE cells treated with 0.25 μM niclosamide for 24 hours. The experiment was performed in triplicate. The bars represent means ± SE. Niclosamide treatment (red bar) compared to control (blue bar) (**P* < 0.01). (**B**) Western blot analysis of adherent and NAAE 2LMP cell lines after 24-hour treatment with niclosamide (0, 0.5, 0.25 and 0.125 μM).

Figure 2. The effect of TRA-8, niclosamide and the combination on tumorsphere formation in NAAE cells. NAAE cells were cultured with media alone, niclosamide (0.25 μM), TRA-8 (1 ng/mL 2LMP, 0.5 ng/mL SUM159, 25 ng/mL HCC1143 and 5 ng/mL HCC1187) or the combination for 48 hours. Secondary tumorsphere formation was determined in 4 replicates and 3 separate experiments. **P* < 0.05 treatment vs. control; #*P* < 0.05 combination vs. either agent alone. Additive drug interaction for all four cell lines (*P* = 0.03).

Figure 3. Niclosamide in combination with TRA-8 inhibits Wnt/β-catenin signaling. (**A**) Activity of TCF/LEF plasmid reporter in the TOPflash assay was evaluated in 2LMP, SUM159, HCC1143 and HCC1187 adherent cells. All cell lines were treated with 0.25 μM niclosamide for 48 hours and 0.25 ng/mL TRA-8 for 24 hours. The experiment was performed in triplicate. The bars represent mean ± SE compared to untreated control. Single or combination treatment compared to control (**P* < 0.05), combination treatment vs. either single agent (# *P* < 0.05). (**B**) Western blot analysis of β-catenin degradation after 2-hour treatment with TRA-8 on both adherent and NAAE cell populations of the 2LMP and HCC1187 cell lines. (**C**) LRP6 inhibition effect on TRA-8 cytotoxicity. 2LMP parental, LRP6 KD and shRNA control cells were treated with TRA-8 for 48 hours. The experiment was performed in triplicate. LRP6 KD compared to control shRNA (*P* = 0.01).

Figure 4. Effect of niclosamide and TRA-8 *in vivo* on 2LMP orthotopic tumor growth. Tumors were established in athymic nude mice by MFP implantation of 2 x 10^6 2LMP cells. The therapy started when tumors reached a size of 16 mm^2. Niclosamide (30 mg/kg) was given IP for 21 days, TRA-8 (200

μg) was given IP 2x weekly for 3 weeks. Tumor size was measured with calipers twice a week. Each point in the curve represents the mean ± SE (n=5). Single agent niclosamide or TRA-8 vs. control (*P < 0.05), combination treatment vs. control (**P < 0.01), combination treatment vs. TRA-8 or niclosamide (#P < 0.05).

Figure 5. Metastatic pleural effusion patient sample sensitivity to niclosamide alone and in combination with TRA-8. (**A**) Sensitivity of patient samples to 48 hour niclosamide mediated cytotoxicity (1, 2, 4, and 8 μM) compared to untreated controls. Individual experiments were assayed in quadruplicate and bars represent mean ± SE (P < 0.01). (**B**) Activity of TCF/LEF viral reporter in the TOPflash assay was evaluated for patient samples UAB03 and UAB05. Both samples were treated with 4 μM niclosamide for 24 hours in triplicate. The bars represent mean ± SE. Niclosamide compared to control (*P < 0.05). (**C**) Western blot analysis of patient sample UAB03 after 24 hour treatment with niclosamide (4 μM). (**D**) Cytotoxicity of patient samples UAB03 and UAB05 pre-treated with niclosamide for 24 hours followed by 24 hours with TRA-8. Cell viability was analyzed using ATPlite assay.

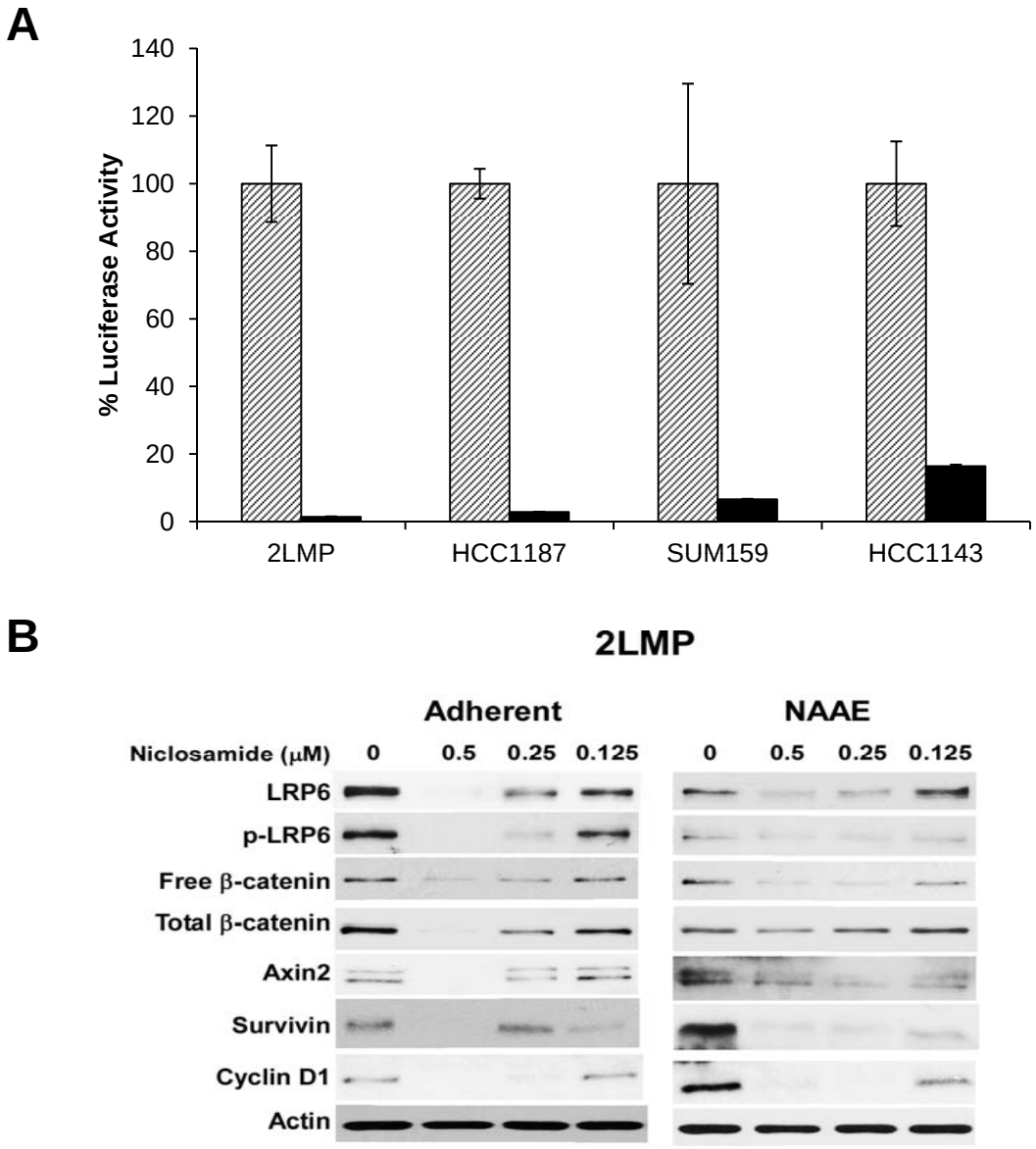
A
140
120
100
80
60
40
20
0
% Luciferase Activity
2LMP
HCC1187
SUM159
HCC1143
B
2LMP
Adherent
NAAE
Niclosamide (μM)
0
0.5
0.25
0.125
0
0.5
0.25
0.125
LRP6
p-LRP6
Free β-catenin
Total β-catenin
Axin2
Survivin
Cyclin D1
Actin

Figure 1

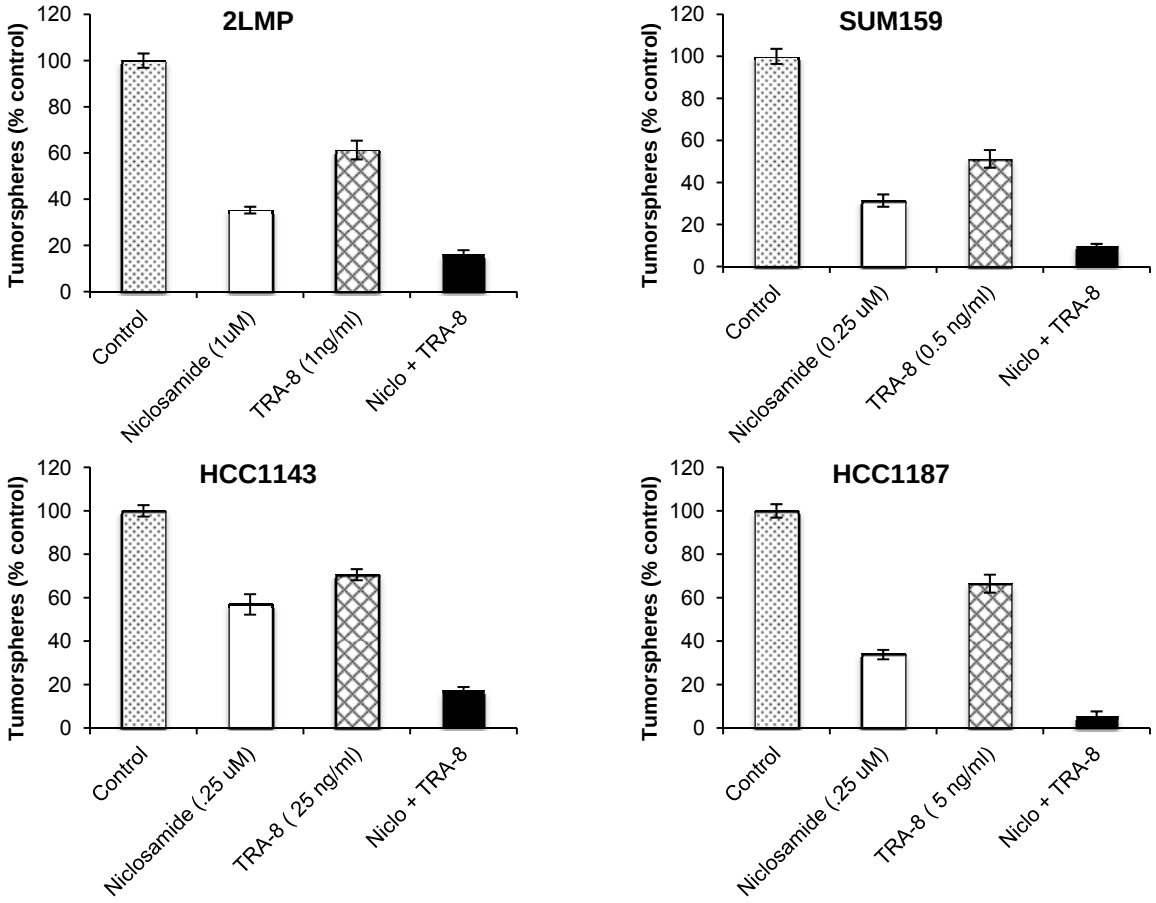
2LMP
Tumorspheres (% control)
120
100
80
60
40
20
0
Control
Niclosamide (1uM)
TRA-8 (1ng/ml)
Niclo + TRA-8
SUM159
Tumorspheres (% control)
Control
Niclosamide (0.25 uM)
TRA-8 (0.5 ng/ml)
Niclo + TRA-8
HCC1143
Tumorspheres (% control)
Control
Niclosamide (.25 uM)
TRA-8 (25 ng/ml)
Niclo + TRA-8
HCC1187
Tumorspheres (% control)
Control
Niclosamide (.25 uM)
TRA-8 (5 ng/ml)
Niclo + TRA-8

Figure 2

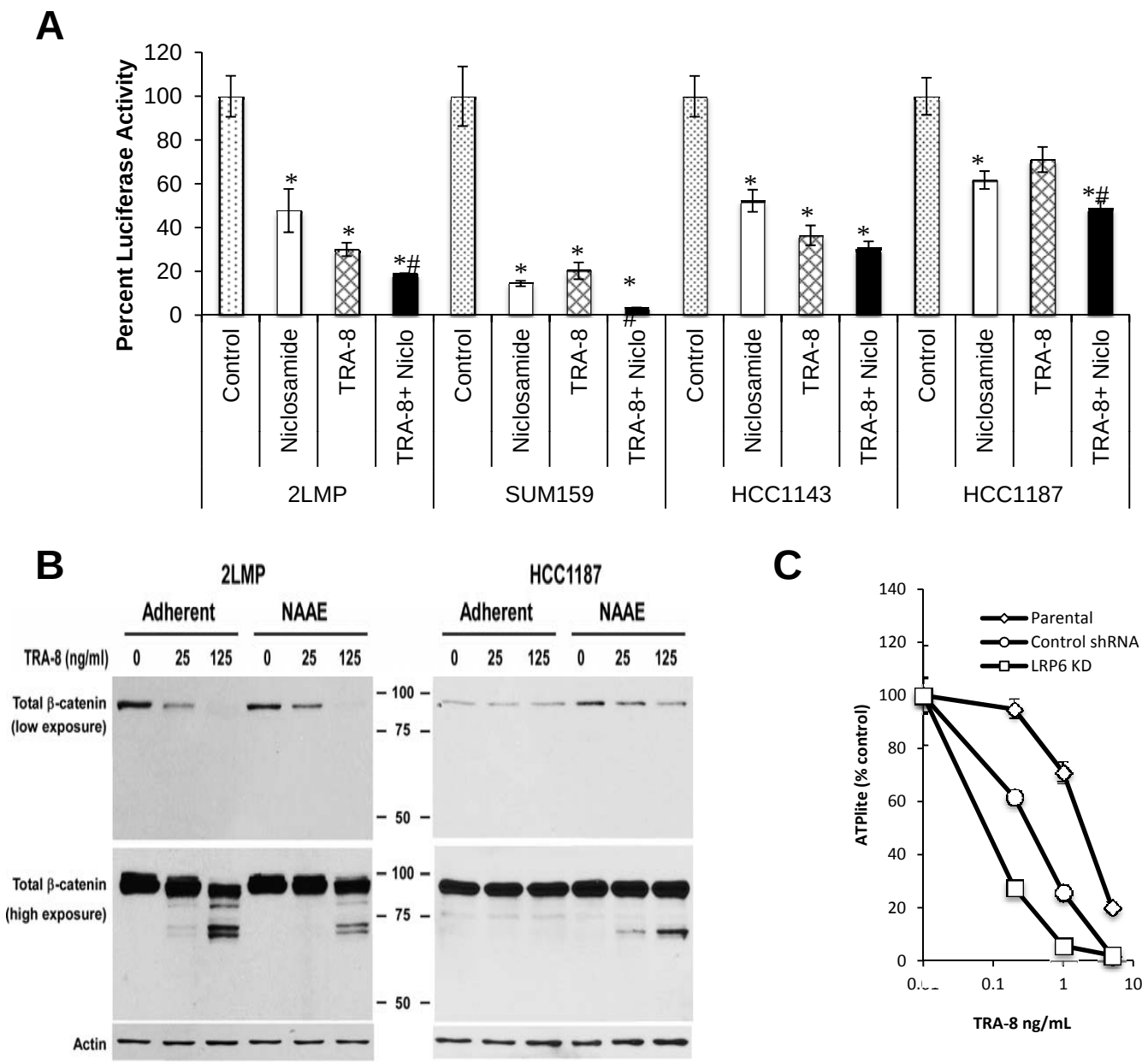
A
Percent Luciferase Activity
120
100
80
60
40
20
0
Control
Niclosamide
TRA-8
TRA-8+ Niclo
2LMP
SUM159
HCC1143
HCC1187
B
2LMP
HCC1187
Adherent
NAAE
TRA-8 (ng/ml)
0
25
125
Total β-catenin
(low exposure)
Total β-catenin
(high exposure)
Actin
100
75
50
C
Parental
Control shRNA
LRP6 KD
ATPlite (% control)
TRA-8 ng/mL
0.1
1
10

Figure 3

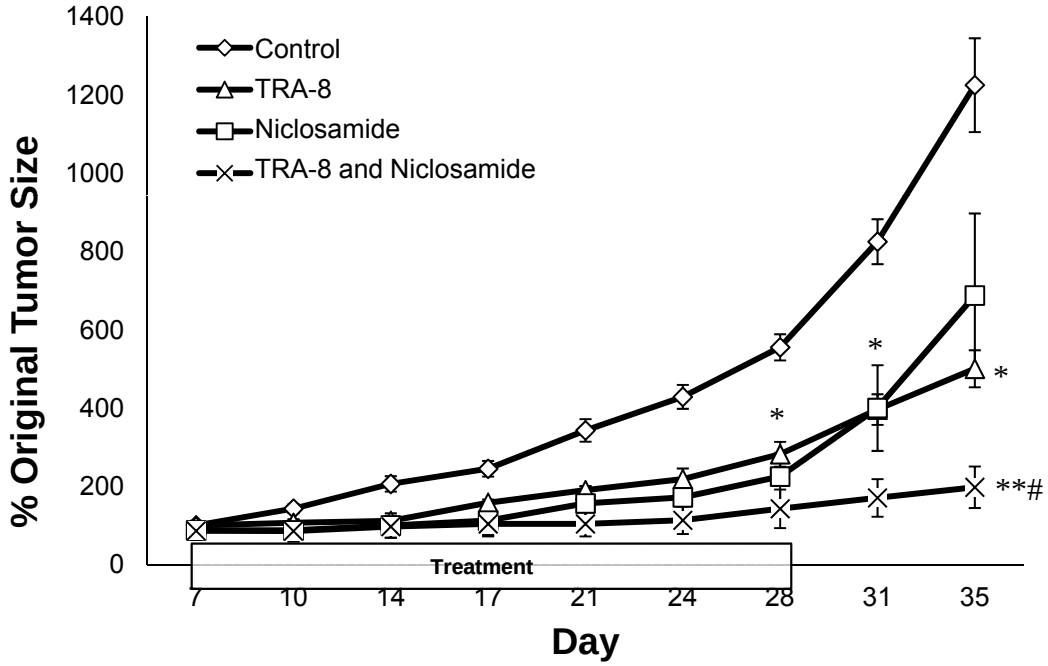
1400
1200
1000
800
600
400
200
0
% Original Tumor Size
Control
TRA-8
Niclosamide
TRA-8 and Niclosamide
Treatment
7
10
14
17
21
24
28
31
35
Day
*
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Figure 4

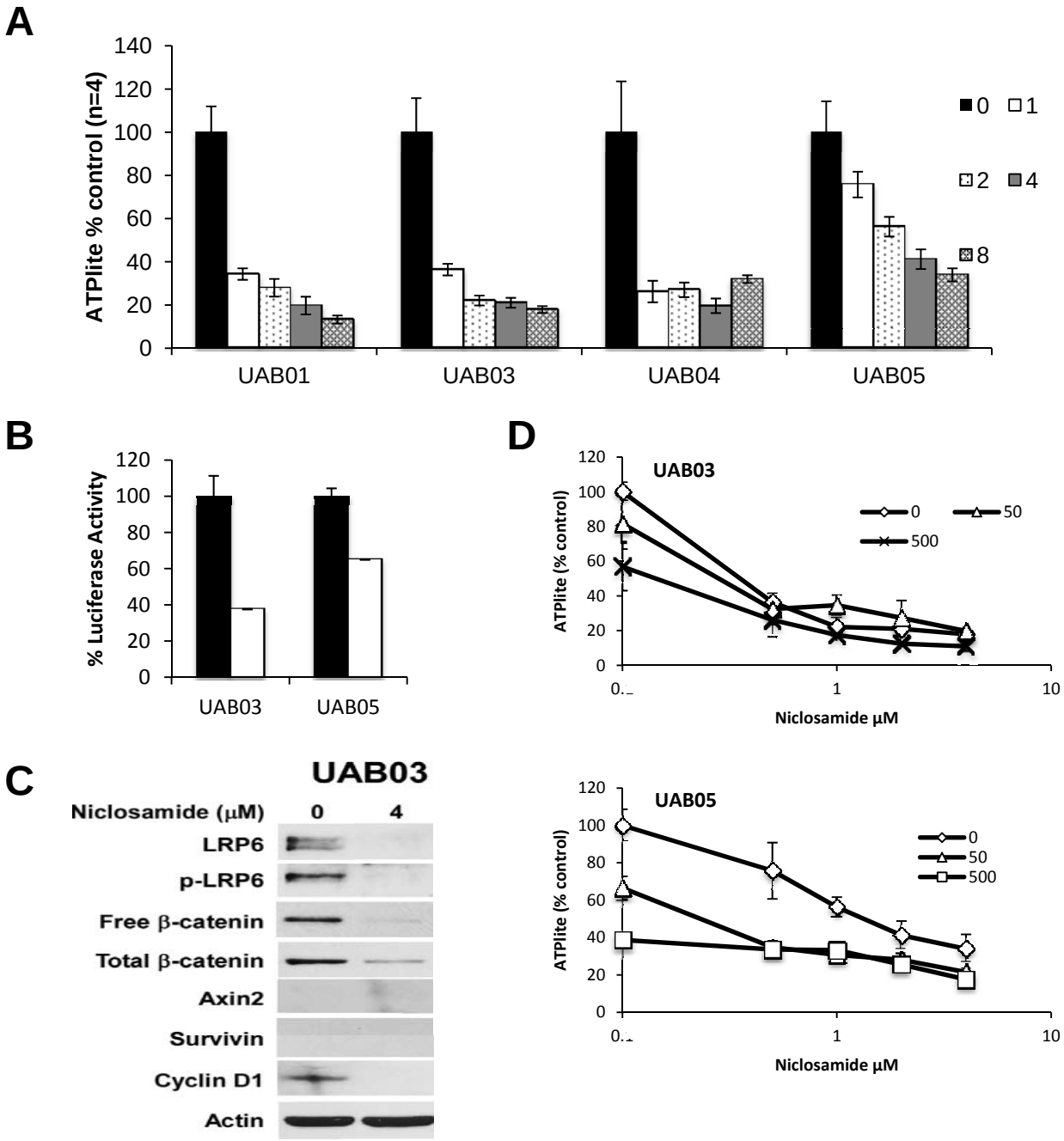
A
ATPlite % control (n=4)
140
120
100
80
60
40
20
0
0
1
2
4
8
UAB01
UAB03
UAB04
UAB05
B
% Luciferase Activity
120
100
80
60
40
20
0
UAB03
UAB05
C
UAB03
Niclosamide (μM)
0
4
LRP6
p-LRP6
Free β-catenin
Total β-catenin
Axin2
Survivin
Cyclin D1
Actin
D
UAB03
ATPlite (% control)
120
100
80
60
40
20
0
0
50
500
1
10
Niclosamide μM
UAB05
ATPlite (% control)
120
100
80
60
40
20
0
0
50
500
1
10
Niclosamide μM

Figure 5

Basal-like breast cancer stem cells are sensitive to anti-DR5 mediated cytotoxicity

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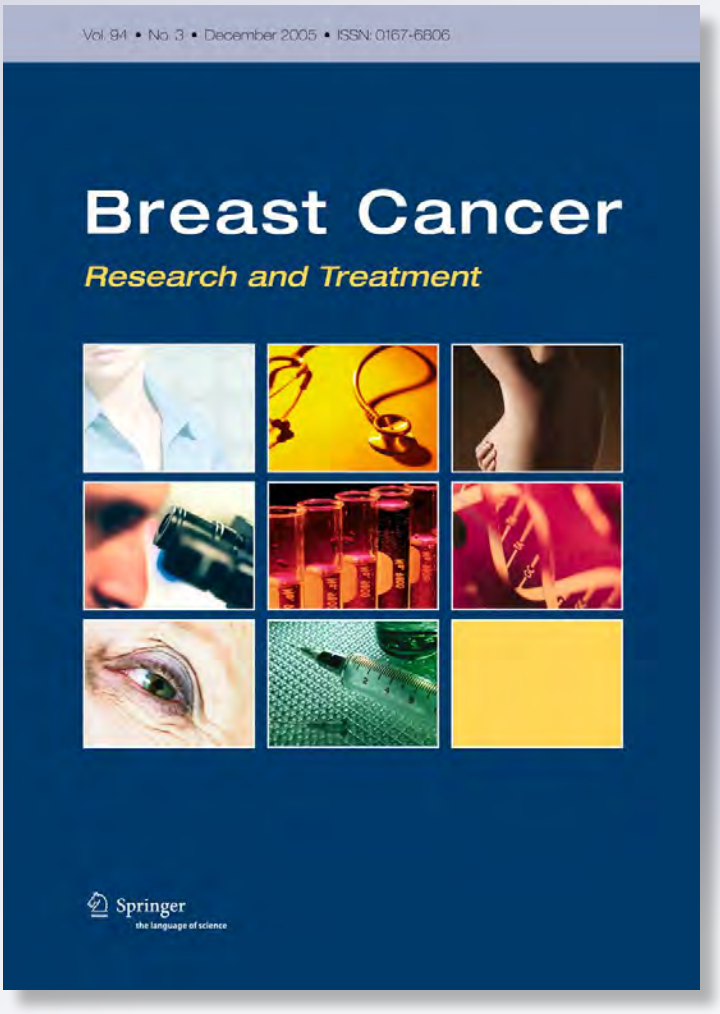

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PRECLINICAL STUDY

Basal-like breast cancer stem cells are sensitive to anti-DR5 mediated cytotoxicity

Angelina I. Londoño-Joshi · Patsy G. Oliver · Yufeng Li · Choo Hyung Lee · Andres Forero-Torres · Albert F. LoBuglio · Donald J. Buchsbaum

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Abstract Breast cancer stem cells (BrCSC) are resistant to common therapeutic modalities including chemotherapy, radiation, and hormonal agents. They are thought to contribute to treatment resistance, relapse, and metastases. This study examines the effect of a monoclonal anti-DR5 antibody (TRA-8) and chemotherapy (adriamycin, taxol) on BrCSC populations from basal-like breast cancer cell lines. Doubly enriched BrCSC ($CD44^+$, $CD24^-$, $ALDH^+$) cells were exposed to TRA-8 and control reagents and examined for cytotoxicity, caspase activation, tumorsphere formation and tumorigenicity. Doubly enriched BrCSC populations expressed cell surface DR5 and were sensitive to TRA-8 mediated cytotoxicity with induction of caspase 8 and 3 activation. TRA-8 at sub-nanomolar concentrations inhibited 2LMP and SUM159 BrCSC tumorsphere formation and was more than 50-fold more inhibitory than TRAIL or anti-DR4 at equimolar concentrations. Chemotherapy treatment of 2LMP and SUM159 cell lines resulted in a relative increase of BrCSC, whereas TRA-8 produced a decrease in the percentage of BrCSC. TRA-8 exposure to 2LMP and SUM159 BrCSC preparations produced significant inhibition of tumorigenicity. DR5 maybe a therapeutic target on the surface of basal-like BrCSC which is amenable to agonistic monoclonal anti-DR5 therapy.

Keywords Anti-DR5 · Tigatuzumab · Basal-like breast cancer · Breast cancer stem cells · Tumor initiating cells · Tumorspheres · Death receptor 5

Introduction

Basal-like breast cancer accounts for about 15% of all breast cancer [1]. It is characterized by a unique mRNA profile with CK5/6 expression, inactivation of *BRCA1* and commonly lacks estrogen receptor, progesterone receptor, and HER-2 amplification [1–3]. They are further categorized into basal A and basal B subtypes and appear to commonly have substantial numbers of breast cancer stem cells (BrCSC) or tumor initiating cells [4–6].

The cancer stem cell hypothesis suggests that tumors, similar to normal tissue, are organized in a cellular hierarchy, with cancer stem cells (CSC) at the top, as the only cells with potentially limitless proliferation abilities which are capable of driving tumor growth [7]. The more 'differentiated' descendants, which account for the majority or bulk of the tumor population, may also be able to proliferate, but regenerative ability is limited [7]. Cancer stem cells were first described in patients with acute leukemia and subsequently in a variety of solid tumors [8, 9]. In breast cancer, CSC were first reported in 2003 by Muhammad Al-Hajj using $CD44^+$ and $CD24^-$ surface expression [10]. Since then BrCSC have been characterized

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based on other cell surface antigens ($EpCAM^+$, $CD133^+$, $CD90^+$) and by functional activities including enhanced efflux pumping of a Hoechst dye (side population), overexpression of aldehyde dehydrogenase (ALDH, ALDEFLUOR assay), retention of the lipophilic dye PKH26, and tumorsphere-forming ability [11–14]. BrCSC are also called tumor initiating cells that are described as having the ability to self-renew, induce tumors at low cell numbers, have low rates of cell division, exhibit chemotherapy and radiation resistance, and have gene expression profiles which differ from the more differentiated cancer cell counterparts [15]. The concept of solid tumor and particularly BrCSC is controversial with several alternative explanations for stem-like cell behaviors [11, 16].

CSC are generally reported to be resistant to chemotherapy and radiation and BrCSC commonly lack "targetable" receptors like ER or HER2 [17–19]. Thus, there is considerable interest in finding therapeutic agents targeted to BrCSC. The presence of substantial numbers of BrCSC in basal-like breast cancer cell lines [10] provided the opportunity to examine the effects of TRA-8 (anti-DR5) on BrCSC enriched populations in terms of anti-DR5 mediated cytotoxicity, inhibition of tumorsphere formation in vitro, and tumorigenicity in vivo. TRA-8 is an agonistic monoclonal anti-DR5 antibody with cytotoxicity and antitumor activity in a variety of human tumor cell lines and murine tumor xenografts [20–23] including basal-like breast cancer cell lines [24].

Materials and methods

Drugs and antibodies

Adriamycin and taxol were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) and prepared as 10 mM stock solutions in distilled H_2O or DMSO, respectively. Purified TRA-8 (IgG1) mAb was provided by Tong Zhou at the University of Alabama at Birmingham (UAB) as described previously [25]. Isotype-specific IgG1 control antibody was obtained from Southern Biotechnology Associates (Birmingham, AL). Anti-DR4 mAb 2E12 (IgG1, k) was provided by Tong Zhou (UAB). Super Killer TRAIL™ was purchased from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). Conjugated antibodies APC mouse anti-human CD44, PE-Cy7 rat anti-mouse CD44, and corresponding isotype control antibodies were purchased from BD Pharmingen (San Jose, CA). ALDEFLUOR kit including diethylaminobenzaldehyde (DEAB) negative control was obtained from StemCell Technologies (Durham, NC). Cleaved caspase 8 rabbit mAb and cleaved caspase 3 rabbit mAb were purchased from Cell Signaling (Billerica, MA). Secondary antibodies, Alexa fluor 405 goat anti-rabbit IgG, and Alexa fluor 647 goat anti-mouse IgG1 were purchased from Invitrogen (Carlsbad, CA).

Cells and cell culture

The 2LMP subclone of the human breast cancer cell line MDA-MB-231 was obtained from Dr. Marc Lippman (University of Miami, Coral Gables, FL) and maintained in improved MEM supplemented with 10% FBS (Hyclone, Logan, UT). Basal-like cell lines HCC38, HCC1187, HCC1143, MDA-MB-436, BT-20, and BT-549 were obtained from American Type Culture Collection (Manassas, VA) and cultured according to supplier's directions with the exception of MDA-MB-436, which was grown in DMEM supplemented with 10 μg/ml insulin, glutathione, and 10% FBS. SUM159 was obtained from Asterand (Detroit, MI) and grown according to supplier's recommendation. All cell lines were maintained in antibiotic-free medium at 37°C in a 5% CO_2 atmosphere and routinely screened for Mycoplasma contamination. Sorted cells and tumorspheres were maintained in MEGM medium (Lonza, Walkersville, MD).

Doubly enriched BrCSC isolation by flow cytometry

Basal-like cell lines were plated in T75 flasks (Costar, Cambridge, MA) in corresponding media and harvested at 75% confluence. Cells were harvested with trypsin and labeled with 1 μl of ALDEFLUOR reagent in 100 μl ALDEFLUOR buffer per 5×10^6 cells and incubated at 37°C for 30 min. Cells were then labeled with APC-CD44 (1:25) and PE-CD24 (1:25) in 200 μl of ALDEFLUOR buffer on ice for 15 min. The ALDEFLUOR positive population was established by using 2×10^6 ALDEFLUOR labeled cells and 5 μl DEAB in 200 μl ALDEFLUOR buffer. The sorting gates were established using negative controls, DEAB and side scatter and forward scatter profiles were used to eliminate cell doublets [10, 11, 16]. Samples were sorted on a Becton–Dickinson-FACSAriaII™ or analyzed on Becton–Dickinson-LSRII™ flow cytometer (Chicago, IL). Data was evaluated using FlowJo software (Tree Star, Inc., Ashland, OR).

DR5 expression and functional caspase activation

2LMP, SUM159, and HCC1143 cell lines were harvested using cell stripper (Mediatech, Manassas, VA) to prevent cleavage of death receptor. Cells were incubated with ALDEFLUOR reagents for 30 min at 37°C. Cells were then labeled on ice with TRA-8 (IgG1) or IgG1 istotype control for 15 min. Cells were then incubated with CD44-PE-Cy7 (1:1,000), CD24-PE (1:100), and secondary

antibody (Alexa-647) (1:100) for 15 min on ice. Samples were analyzed by flow cytometry for DR5 expression on the $CD44^+/CD24^-/ALDH^+$ subpopulation. Analysis of caspase 8 and 3 activation of BrCSC was accomplished by harvesting cells using cell stripper and sorting for the $ALDH^+$ population. Sorted cells were treated for 2 h with TRA-8 or IgG1 control ($\sim 1 \times 10^6$ cells with 1 μg/ml TRA-8 or IgG1 in MEGM medium + 2% BSA). Cells were fixed with 1% paraformaldehyde for 5 min on ice and labeled with CD44-APC and CD24-PE (1:100) on ice for 15 min. Cells were then permeabilized using 3% BSA, 0.1% saponin in 200 μl PBS on ice for 15 min and labeled with cleaved caspase 3 or 8 (1:500) on ice for 15 min. Cells were incubated with secondary antibody Alexa-405 anti-rabbit (1:100) on ice for 15 min. Samples were kept in 0.1% saponin and analyzed by flow cytometry. Analysis was done on a minimum of three independent experiments.

Cell viability assays using ATPLite

Sorted $CD44^+/CD24^-/ALDH^+$ cells were plated on ultra-low attachment plates (Costar) at 2,000 cells per 50 μl of MEGM medium. Bulk unseparated cells were collected from total viable gates established by forward and side scatter parameters to control for any variables introduced by sorting the cells. Cells from the bulk unseparated populations were plated in optically clear 96-well black plates (Costar) in corresponding media. Sorted and bulk cells were treated with (0.1, 1, 10, 100, or 1,000 ng/ml) of TRA-8 immediately after plating and incubated for 24 h at 37°C. TRA-8 was diluted in culture medium immediately before use. Cell viability was determined by measurement of cellular ATP levels using the ATPLite luminescence-based assay (Packard Instruments, Meriden, CT) described elsewhere [26]. The manufacturer's recommended protocol was followed with the exception that all reaction volumes (culture medium and reagents) were reduced by one-half. All samples were assayed in quadruplicate and IC_{50} values are reported as the median from a minimum of three independent experiments.

In vitro treatment of tumorspheres

2LMP and SUM159 cell lines were sorted for $ALDH^+$ cells. Approximately $\sim 1 \times 10^6$ cells were allowed to form primary spheres at a density of 100,000 cells/ml for 3–4 days in MEGM medium. Tumorspheres were mechanically dissociated and plated in ultra-low attachment 96-well plates (Costar) at 2,000 cells per well. TRA-8 (anti-DR5), 2E12 (anti-DR4), TRAIL, IgG isotype control, adriamycin, or taxol were added to the wells and incubated at 37°C for 48 h in quadruplicate. Tumorspheres were visually counted using a reticle eye piece. Mean tumorsphere inhibition was calculated relative to untreated control spheres. At least three independent experiments were conducted per cell line in quadruplicate.

Effect of drug treatment of breast cancer cells on BrCSC population

2LMP and SUM159 breast cancer cells were plated in 6-well well culture plates at 80,000 cells/well (Costar 3516). Cells were treated with adriamycin (200 nM) or taxol (200 nM) for 48 h, or TRA-8 (10 ng/ml) for 24 h. Cells in suspension after treatment along with attached cells were harvested and incubated with Aldefluor reagent for 30 min at 37°C following manufacturer's protocol. Cells were analyzed using a LSRII™ flow cytometer (Becton–Dickinson). Cells were gated based on forward and side scatter properties for single viable cells. The signal from autofluorescence of drug treatment was accounted for in the final analysis of BrCSC ALDH marker expression.

Ex vivo treatment of BrCSC and tumor implantation

$CD44^+/CD24^-/ALDH^+$ 2LMP and SUM159 cells (1×10^6) were sorted and allowed to recover for 13 h in MEGM medium in ultra-low attachment plates at 37°C. Cells (2×10^4) were separated into treatment groups and drug or antibody was added (IgG, 20 nM), 2E12 (20 nM), TRA-8 (20 nM), and adriamycin (500 nM). Cells were treated for 3 h at 37°C and then aliquoted in 200 μl (1:1 Matrigel) and injected into the mammary fat pad of 4 week old NOD/SCID mice (Harlan, Prattville, AL). Tumor size was determined by the product of two largest diameters. Two independent animal experiments were conducted for the 2LMP and SUM159 cell lines.

Statistical analysis

The IC_{50} is the drug concentration producing the median effect of 50% cell killing which was estimated based on the Hill Equation with nonlinear regression model for each assay [27]. Due to small number of replicate experiments, a nonparametric statistical method with Kruskal–Wallis test was used for the comparison between two groups, e.g., IC_{50}, percentage of tumorsphere number and ATP level [28]. The Generalized Linear Model (GLM) with PROC MIXED was used to compare the tumor size over time among experimental groups. Main effect and interaction between treatment groups and measurement time point were fitted in the model with appropriate variance and covariance structure selected. The statistical analysis was carried out with Statistical Analysis Software (SAS) version 9.2.

Results

Anti-DR5 (TRA-8) induced cytotoxicity to BrCSC enriched cells

Eight basal-like cell lines underwent dual BrCSC enrichment ($CD44^+/CD24^-/ALDH^+$) and these BrCSC enriched populations were compared with their unseparated parental cells in regard to sensitivity to anti-DR5 (TRA-8) mediated cytotoxicity (Table 1). As reported previously [24], the basal-like cell lines were quite sensitive ($IC_{50} < 100$ ng/ml) to TRA-8 mediated cytotoxicity except for HCC1143 which was moderately resistant (IC_{50} of 101–1,000 ng/ml). All the cell line BrCSC enriched cell preparations were very sensitive to anti-DR5 mediated cytotoxicity including cell line HCC1143. In 6/8 instances, the BrCSC enriched cells were significantly more sensitive than their parental cells.

DR5 expression of BrCSC enriched cells

The dual separated ($CD44^+/CD24^-/ALDH^+$) and unseparated cell preparations from 2LMP, SUM159, and HCC1143 underwent flow cytometry for analysis of cell membrane expression of DR5. As shown in Fig. 1, cells from all cell lines were strongly positive and the unseparated (blue lines) and dual enriched (red lines) had comparable expression of DR5.

Apoptosis of anti-DR5 treated BrCSC enriched cell populations

To determine that anti-DR5 can mediate apoptosis of the BrCSC enriched cells, the 2LMP and SUM159 $ALDH^+$ populations were incubated with anti-DR5 or control IgG for 2 h, and the $CD44^+$ $CD24^-$ cell population were tested for cellular expression of activated caspase 8 and activated caspase 3. Figure 2 illustrates that a substantial portion of the 2LMP (Fig. 2a) and SUM159 (Fig. 2b) BrCSC underwent caspase 8 and 3 activation (green lines) as compared with BrCSC exposed to control IgG (red line). This delineates that anti-DR5 triggers caspase activation and apoptosis of BrCSC doubly enriched cells over even short durations of 2–3 h.

Analysis of anti-DR5 effect on BrCSC tumorsphere formation

Tumorsphere formation has been reported as a measure of BrCSC presence in enriched cell populations [29]. Figure 3 provides the effects of anti-DR5 (TRA-8), anti-DR4 (2E12), TRAIL, adriamycin, taxol, control IgG, and control media on secondary tumorsphere formation of 2LMP (Fig. 3a), and SUM159 (Fig. 3b) cell lines. Impressive inhibition of secondary tumorsphere formation was caused by anti-DR5 at doses as low as 0.1 nM with 80% inhibition of 2LMP cells ($P = 0.019$) and 95% inhibition of SUM159 cells ($P = 0.02$). Anti-DR5 produced significantly more inhibition of tumorsphere formation at 0.1 nM than 5.0 nM TRAIL ($P = 0.019$) or 5.0 nM anti-DR4 ($P = 0.028$) in 2LMP cells. The SUM159 cells had comparable observations with 0.1 nM anti-DR5 producing more inhibition than 5.0 nM TRAIL ($P = 0.019$) or 5.0 nM anti-DR4 ($P = 0.02$). Adriamycin and taxol had modest or no inhibitory effects. Thus, anti-DR5 appears to be superior to other DR-mediated agents at tumorsphere formation inhibition.

Effect of drug exposure of breast cancer cell lines on BrCSC population

Drug or TRA-8 treatment of breast cancer cell lines may change the percentage of $ALDH^+$ cells (BrCSC) in the total cell population. Flow cytometry analysis of 2LMP basal-like cells after adriamycin or taxol treatment showed a 4.6-fold or 2.2-fold increase of $ALDH^+$ cells, respectively (Fig. 4). SUM159 cells treated with adriamycin or taxol had a 3.3-fold and 1.9-fold increase in the percentage

Table 1 Sensitivity of sorted BrCSC and unseparated parental cells to TRA-8 mediated cytotoxicity

Phenotype	Cell line	Sorted $CD44^+CD24^-$ $ALDH^+$ IC_{50} TRA-8 (ng/ml)	Unseparated parental IC_{50} TRA-8 (ng/ml)	*P* value sorted vs. unsorted
Basal B	HCC38	0.10[a]	0.74	0.127
	2LMP	0.65	1.06	0.008
	SUM159	0.88	5.54	0.049
	MDA-MB-436	0.62	0.31	0.248
	BT-549	0.63	5.55	0.02
Basal A	HCC1187	0.85	24.72	0.049
	BT-20	7.24	16.49	0.275
	HCC1143	77.12	628.43	0.049

[a] All samples were assayed in quadruplicate and are reported as the median from a minimum of three independent experiments

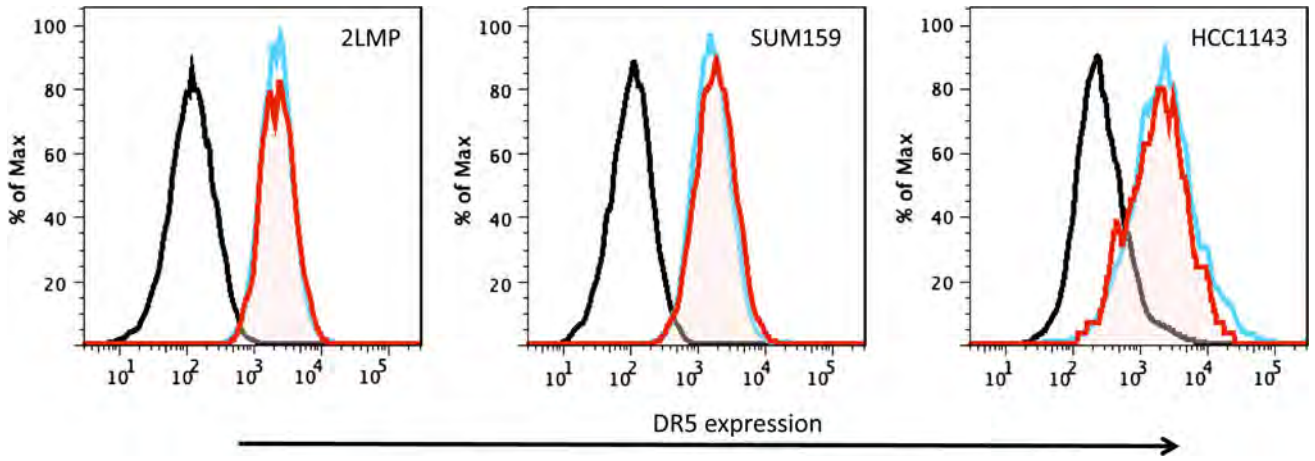

Fig. 1 Flow cytometry analysis of DR5 membrane expression on the $CD44^+/CD24^-/ALDH^+$ subpopulation of basal-like breast cancer cells. 2LMP, SUM159, and HCC1143 cells were labeled with ALDEFLUOR (FITC), CD44 (PE, Cy7), CD24 (PE), and anti-DR5 (TRA-8 Alexa-647) then analyzed by flow cytometry. DR5 membrane expression on total unsorted bulk population (*blue line*) or $CD44^+/CD24^-/ALDH^+$ (*red line*) subpopulation compared with isotype control (*black line*). 2LMP, SUM159, and HCC1143 $CD44^+/CD24^-/ALDH^+$ subpopulations had similar DR5 expression compared with the total unsorted cell population

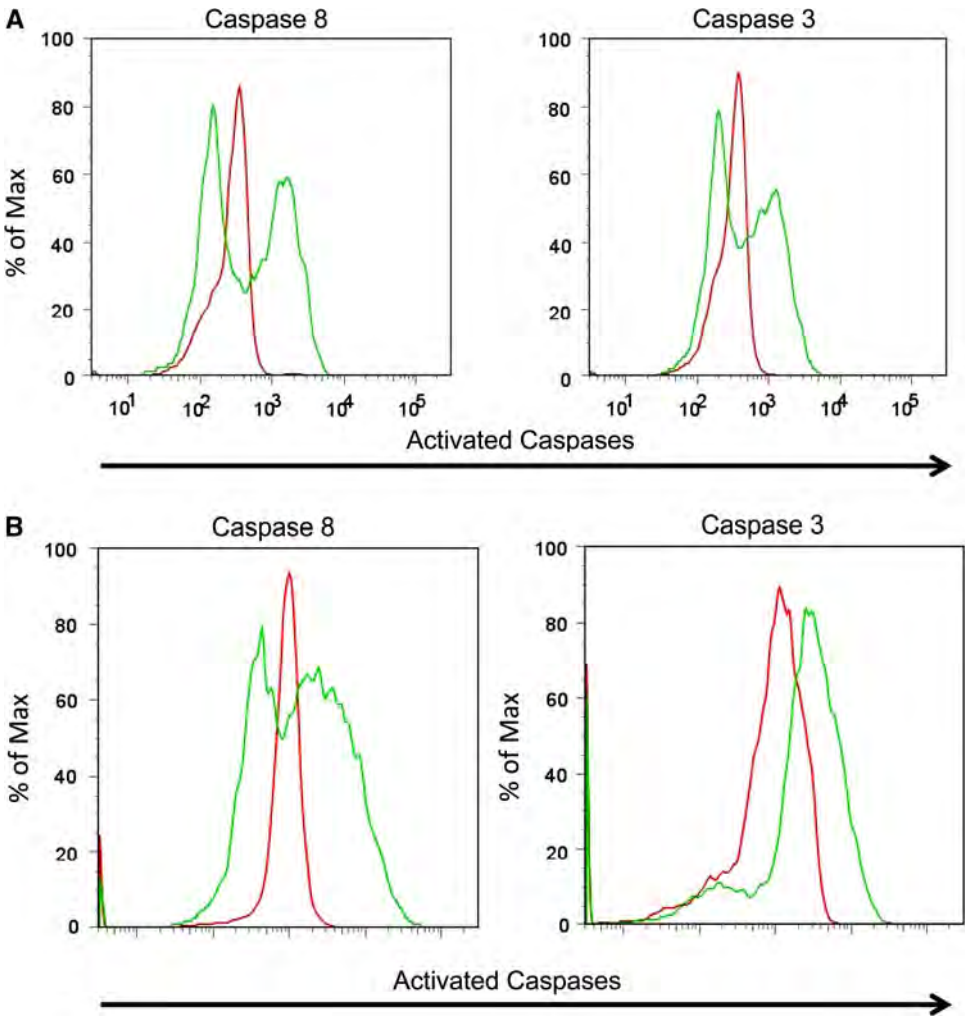

Fig. 2 Flow cytometry analysis of caspase activation in $CD44^+/CD24^-/ALDH^+$ subpopulation of 2LMP and SUM159 cells after treatment with TRA-8. 2LMP (**a**) and SUM159 (**b**) cells were sorted for the $ALDH^+$ subpopulation and then treated with TRA-8 or control IgG for 2 h. Cells were then fixed and stained for CD44 (APC), CD24 (PE), and activated caspases 3 or 8 (secondary Alexa 405). 2LMP and SUM159 BrCSC enriched cells had caspase 3 and caspase 8 activation (*green line*) after incubation with TRA-8 compared with IgG control (*red line*)

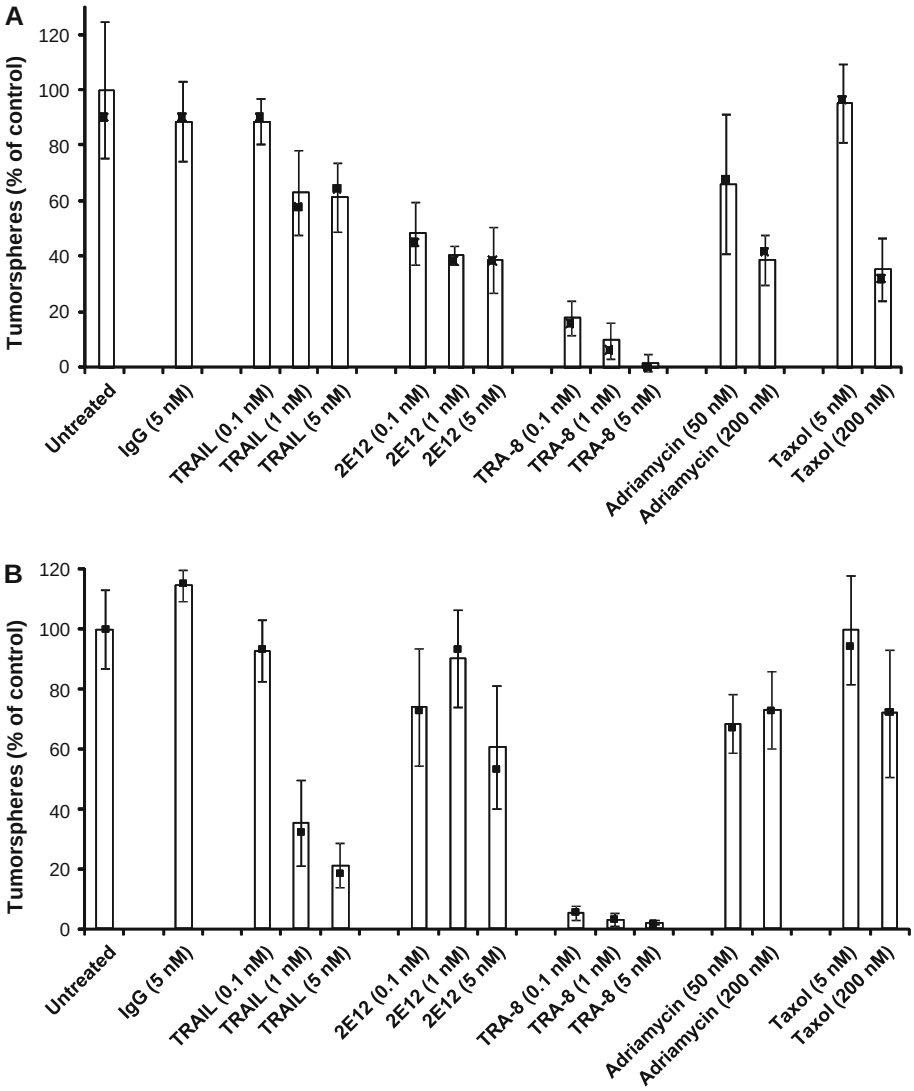

Fig. 3 Secondary tumorsphere formation inhibition by TRA-8. 2LMP cells (**a**) and SUM159 (**b**) cells were sorted using flow cytometry for ALDH$^+$ cells and allowed to form primary tumorspheres for 3 days. After tumorspheres were mechanically dissociated, single cells (2,000 cells/well) were plated in low attachment plates and treated with IgG, TRAIL, 2E12 (anti-DR4), TRA-8, adriamycin or taxol. After 48 h, tumorspheres ranging from 40 to 120 μm in size were visually counted using a reticle eye piece. Mean tumorsphere inhibition was calculated relative to untreated controls (*blue bars*) (*filled square*) represent median values. *Error bars* represent SD of the samples run in quadruplicate

of ALDH$^+$ cells, respectively. By contrast, 2LMP and SUM159 cells treated with TRA-8 resulted in a 1.8-fold and 1.7-fold decrease in ALDH$^+$ tumor cells compared with untreated cells, respectively. Thus, BrCSC appear to be resistant to chemotherapy but sensitive to anti-DR5.

Effect of anti-DR5 exposure on BrCSC enriched cell population tumorigenicity

The major requirement of BrCSC is the ability to generate fully constituted human breast cancer in immuno-compromised mice. BrCSC enriched cell populations were exposed to anti-DR5 (TRA-8), anti-DR4 (2E12), control IgG, or adriamycin for 3 h before injection of treated cells into the mammary fat pads of NOD/SCID mice ($n = 5$ for each group). Figure 5a illustrates that the control IgG, anti-DR4, and adriamycin treated cells generated 5/5 tumors. The IgG control mice were sacrificed on day 44 to comply

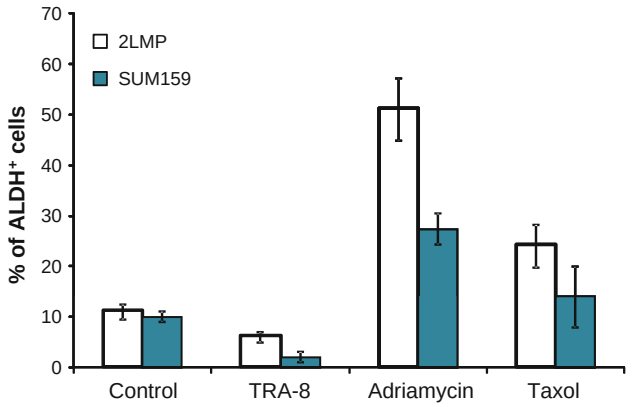

Fig. 4 In vitro treatment with TRA-8 decreased the percentage of ALDH$^+$ cells in 2LMP and SUM159 breast cancer cell lines. 2LMP and SUM159 cells (80,000 cells/well) were treated with TRA-8 (10 ng/ml) for 24 h, and adriamycin (200 nM) or taxol (200 nM) for 48 h in 6-well cell culture plates. Mean ALDH$^+$ cells after treatment from a minimum of three independent experiments are shown. *Error bars* denote SE

with IACUC guidelines with tumor sizes exceeding 175 mm^2. The anti-DR4 and adriamycin treated tumors were somewhat slower growing but not statistically different than the control. In contrast, the anti-DR5 treated cells developed measurable tumors in only 2/5 animals by day 50 and even these tumors were small and had severely retarded growth compared with the IgG control treated tumors ($P = 0.0001$). Figure 5b illustrates examples of the 2LMP tumors in the control and treated NOD/SCID mice. The tumors that grew in the four treated groups of mice had similar histology with poorly differentiated cells, high mitotic rates, and focal areas of necrosis. Similar studies with SUM159 cell line showed that the IgG treated BrCSC enriched cells generated 5/5 tumors compared with 0/5 mice observed in the TRA-8 treated group at day 105. Thus, it appears that TRA-8 can seriously impair the tumorigenicity of BrCSC enriched cell populations.

Discussion

There is considerable interest in finding therapeutic agents that could be targeted to CSC to enhance the efficacy of treatment regimens and potentially reduce tumor resistance and relapse. We have previously shown the anti-tumor activity of an agonistic monoclonal anti-DR5 antibody (TRA-8) to 2LMP and other basal-like cell lines in vitro and in vivo [24]. Others have shown DR-mediated cytotoxicity to basal B but not basal A breast cancer cell lines [30]. Given that basal-like cell lines are enriched in CSC [5, 18], it represented an opportunity to examine the effect of TRA-8 on BrCSC populations.

Doubly enriched BrCSC subpopulations ($CD44^+/CD24^-/ALDH^+$) of both basal-like A and B type were sensitive to TRA-8 mediated cytotoxicity. Further, DR5 expression on BrCSC subpopulations were identical to their unseparated parental population and brief interaction with TRA-8 triggered caspase 8 and 3 activation. Thus, it appears that basal-like BrCSC subpopulations share sensitivity to anti-DR5 mediated cytotoxicity similar to their parental cells and in some instances even have increased sensitivity. In vitro treatment of parental breast cancer cell lines with adriamycin or taxol increased the percentage of $ALDH^+$ cells, while TRA-8 produced a decrease in the percentage of $ALDH^+$ cells. These results indicate that the bulk cells were more sensitive to chemotherapy treatment than the BrCSC, whereas the BrCSC were more sensitive to TRA-8 treatment than the bulk cells. Similarly, anti-DR5 treatment decreased the percentage of CSC in pancreatic cancer [31].

A prior study had reported that TRAIL was able to mediate cytotoxicity to colon cancer CSC (dye efflux side population) and that this population was enriched for expression of DR4 [32]. We thus contrasted the effects of TRAIL, anti-DR4, and anti-DR5 on BrCSC tumorsphere formation. These studies demonstrated the superiority of TRA-8 over TRAIL and anti-DR4 in terms of inhibition of BrCSC tumorsphere formation. Similarly, TRA-8 was

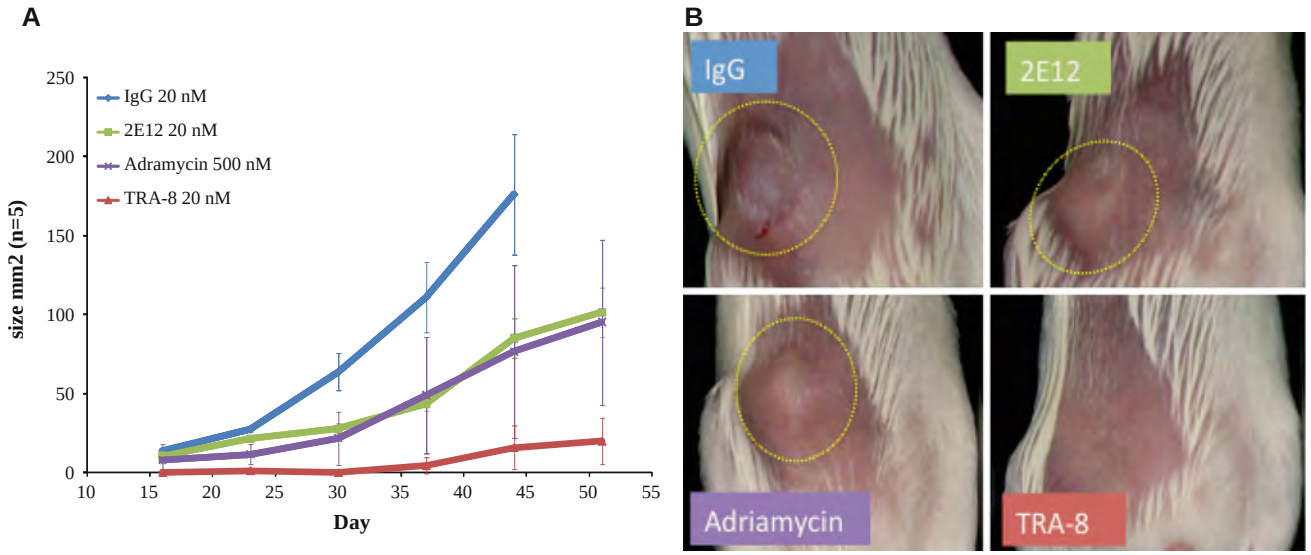

Fig. 5 Effect of ex vivo treatment of BrCSC enriched cells on tumorgenicity in NOD/SCID mice. 2LMP cells were sorted using flow cytometry for $CD44^+/CD24^-/ALDH^+$ BrCSC markers and the cells were allowed to recover for 13 h. Cells were treated with TRA-8, 2E12, adriamycin or IgG control for 3 h and implanted into the MFP of groups of five NOD/SCID mice. **a** *Graph* represents the average tumor size and number of tumors formed. Only 2/5 small, slow growing tumors were observed to develop within 50 days with TRA-8 treated cells while 5/5 tumors developed in the IgG, 2E12, and adriamycin treatment groups (P value <0.0001). **b** These are images taken of one representative mouse in each group at day 30 after implantation (*dotted circle* shows the tumor)

superior to anti-DR4 in inhibition of BrCSC tumorigenicity. This may reflect differences among CSC of different tumor types. Indeed, CSC from glioblastoma cell lines have been reported to be resistant to TRAIL mediated cytotoxicity [33].

These observations suggest that DR5 maybe a target on the surface of basal-like breast cancer cell lines and BrCSC by which an agonistic monoclonal anti-DR5 antibody could mediate anti-tumor activity/efficacy. Tigatuzumab is the CDR grafted, humanized version of TRA-8 which has entered clinical trials [34]. Because of these studies and others, the Translational Breast Cancer Research Consortium has recently opened a randomized phase II trial of abraxane ± tigatuzumab for metastatic triple negative breast cancer (ClinicalTrials.gov NCT01307891).

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References

1. Liedtke C, Gonzalez-Angulo A-M, Pusztai L (2010) Definition of triple-negative breast cancer and relationship to basal-like molecular subtype. PPO Updat Prin Prac Oncol 24:1–6
2. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, Hernandez-Boussard T, Livasy C, Cowan D, Dressler L, Akslen LA, Ragaz J, Gown AM, Gilks CB, van de Rijn M, Perou CM (2004) Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res 10(16):5367–5374. doi:10.1158/1078-0432.CCR-04-0220
3. Lakhani SR, Reis-Filho JS, Fulford L, Penault-Llorca F, van der Vijver M, Parry S, Bishop T, Benitez J, Rivas C, Bignon YJ, Chang-Claude J, Hamann U, Cornelisse CJ, Devilee P, Beckmann MW, Nestle-Kramling C, Daly PA, Haites N, Varley J, Lalloo F, Evans G, Maugard C, Meijers-Heijboer H, Klijn JG, Olah E, Gusterson BA, Pilotti S, Radice P, Scherneck S, Sobol H, Jacquemier J, Wagner T, Peto J, Stratton MR, McGuffog L, Easton DF (2005) Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype. Clin Cancer Res 11(14):5175–5180. doi:10.1158/1078-0432.CCR-04-2424
4. Honeth G, Bendahl PO, Ringner M, Saal LH, Gruvberger-Saal SK, Lovgren K, Grabau D, Ferno M, Borg A, Hegardt C (2008) The CD44+/CD24− phenotype is enriched in basal-like breast tumors. Breast Cancer Res 10(3):R53. doi:10.1186/bcr2108
5. Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, Hur MH, Diebel ME, Monville F, Dutcher J, Brown M, Viens P, Xerri L, Bertucci F, Stassi G, Dontu G, Birnbaum D, Wicha MS (2009) Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. Cancer Res 69(4):1302–1313. doi:10.1158/0008-5472.CAN-08-2741
6. Giatromanolaki A, Sivridis E, Fiska A, Koukourakis MI (2010) The CD44+/CD24− phenotype relates to 'triple-negative' state and unfavorable prognosis in breast cancer patients. Med Oncol. doi:10.1007/s12032-010-9530-3
7. Dontu G (2008) Breast cancer stem cell markers—the rocky road to clinical applications. Breast Cancer Res 10(5):110. doi:10.1186/bcr2130
8. Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3(7):730–737
9. Ailles LE, Weissman IL (2007) Cancer stem cells in solid tumors. Curr Opin Biotechnol 18(5):460–466. doi:10.1016/j.copbio.2007.10.007
10. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100(7):3983–3988. doi:10.1073/pnas.0530291100
11. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G (2007) ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 1(5):555–567. doi:10.1016/j.stem.2007.08.014
12. Donnenberg VS, Donnenberg AD, Zimmerlin L, Landreneau RJ, Bhargava R, Wetzel RA, Basse P, Brufsky AM (2010) Localization of CD44 and CD90 positive cells to the invasive front of breast tumors. Cytom B Clin Cytom 78(5):287–301. doi:10.1002/cyto.b.20530
13. Meyer MJ, Fleming JM, Lin AF, Hussnain SA, Ginsburg E, Vonderhaar BK (2010) CD44posCD49fhiCD133/2hi defines xenograft-initiating cells in estrogen receptor-negative breast cancer. Cancer Res 70(11):4624–4633. doi:10.1158/0008-5472.CAN-09-3619
14. Pece S, Tosoni D, Confalonieri S, Mazzarol G, Vecchi M, Ronzoni S, Bernard L, Viale G, Pelicci PG, Di Fiore PP (2010) Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. Cell 140(1):62–73. doi:10.1016/j.cell.2009.12.007
15. Fillmore CM, Kuperwasser C (2008) Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. Breast Cancer Res 10(2):R25. doi:10.1186/bcr1982
16. Croker AK, Goodale D, Chu J, Postenka C, Hedley BD, Hess DA, Allan AL (2009) High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. J Cell Mol Med 13(8B):2236–2252. doi:10.1111/j.1582-4934.2008.00455.x
17. Debeb BG, Xu W, Woodward WA (2009) Radiation resistance of breast cancer stem cells: Understanding the clinical framework. J Mammary Gland Biol Neoplasia 14(1):11–17. doi:10.1007/s10911-009-9114-z
18. Ricardo S, Vieira AF, Gerhard R, Leitao D, Pinto R, Cameselle-Teijeiro JF, Milanezi F, Schmitt F, Paredes J (2011) Breast cancer stem cell markers CD44, CD24 and ALDH1: expression distribution within intrinsic molecular subtype. J Clin Pathol. doi:10.1136/jcp.2011.090456
19. Kakarala M, Wicha MS (2008) Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy. J Clin Oncol 26(17):2813–2820. doi:10.1200/JCO.2008.16.3931
20. DeRosier LC, Vickers SM, Zinn KR, Huang Z, Wang W, Grizzle WE, Sellers JC, Stockard CR Jr, Zhou T, Oliver PG, Arnoletti JP, LoBuglio AF, Buchsbaum DJ (2007) TRA-8 anti-DR5 monoclonal antibody and gemcitabine induce apoptosis and inhibit radiologically validated orthotopic pancreatic tumor growth. Mol Cancer Ther 6:3198–3207. doi:10.1158/1535-7163.MCT-07-0299
21. Oliver PG, LoBuglio AF, Zinn KR, Kim H, Nan L, Zhou T, Wang W, Buchsbaum DJ (2008) Treatment of human colon

cancer xenografts with TRA-8 anti-death receptor 5 antibody alone or in combination with CPT-11. Clin Cancer Res 14:2180–2189. doi:10.1158/1078-0432.CCR-07-1392
22. Amm HM, Zhou T, Steg AD, Kuo H, Li Y, Buchsbaum DJ (2011) Mechanisms of drug sensitization to TRA-8, an agonistic death receptor 5 antibody, involve modulation of the intrinsic apoptotic pathway in human breast cancer cells. Mol Cancer Res 9:403–417
23. Bevis KS, McNally LR, Sellers JC, Della Manna D, Londoño-Joshi A, Amm H, Straughn JJM, Buchsbaum DJ (2011) Anti-tumor activity of an anti-DR5 monoclonal antibody, TRA-8, in combination with taxane/platinum-based chemotherapy in an ovarian cancer model. Gynecol Oncol 121(1):193–199
24. Forero-Torres A, Oliver PG, Joshi AIL, Zhou T, LoBuglio AF, Buchsbaum DJ (2010) Death receptor 5, a therapeutic target for triple negative breast cancer. In: The Breast Cancer Symposium 2010, San Antonio, Texas, 8–12 December 2010
25. Ichikawa K, Liu W, Zhao L, Wang Z, Liu D, Ohtsuka T, Zhang H, Mountz JD, Koopman WJ, Kimberly RP, Zhou T (2001) Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity. Nat Med 7:954–960. doi:10.1038/91000
26. Buchsbaum DJ, Zhou T, Grizzle WE, Oliver PG, Hammond CJ, Zhang S, Carpenter M, LoBuglio AF (2003) Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or radiation therapy in a human breast cancer model. Clin Cancer Res 9:3731–3741
27. Hill AV (1910) The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. J Physiol 40:4–7
28. Gibbons JD (1971) Nonparametric statistical inference. McGraw-Hill, New York
29. Grimshaw MJ, Cooper L, Papazisis K, Coleman JA, Bohnenkamp HR, Chiapero-Stanke L, Taylor-Papadimitriou J, Burchell JM (2008) Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. Breast Cancer Res 10(3):R52. doi:10.1186/bcr2106
30. Rahman M, Davis SR, Pumphrey JG, Bao J, Nau MM, Meltzer PS, Lipkowitz S (2009) TRAIL induces apoptosis in triple-negative breast cancer cells with a mesenchymal phenotype. Breast Cancer Res Treat 113:217–230. doi:10.1007/s10549-008-9924-5
31. Rajeshkumar NV, Rasheed ZA, Garcia-Garcia E, Lopez-Rios F, Fujiwara K, Matsui WH, Hidalgo M (2010) A combination of DR5 agonistic monoclonal antibody with gemcitabine targets pancreatic cancer stem cells and results in long-term disease control in human pancreatic cancer model. Mol Cancer Ther 9(9):2582–2592. doi:10.1158/1535-7163.MCT-10-0370
32. Sussman RT, Ricci MS, Hart LS, Sun SY, El-Deiry WS (2007) Chemotherapy-resistant side-population of colon cancer cells has a higher sensitivity to TRAIL than the non-SP, a higher expression of c-Myc and TRAIL-receptor DR4. Cancer Biol Ther 6(9):1490–1495
33. Capper D, Gaiser T, Hartmann C, Habel A, Mueller W, Herold-Mende C, von Deimling A, Siegelin MD (2009) Stem-cell-like glioma cells are resistant to TRAIL/Apo2L and exhibit down-regulation of caspase-8 by promoter methylation. Acta Neuropathol 117(4):445–456. doi:10.1007/s00401-009-0494-3
34. Forero-Torres A, Shah J, Wood T, Posey J, Carlisle R, Copigneaux C, Luo FR, Wojtowicz-Praga S, Percent I, Saleh M (2010) Phase I trial of weekly tigatuzumab, an agonistic humanized monoclonal antibody targeting death receptor 5 (DR5). Cancer Biother Radiopharm 25:13–19. doi:10.1089/cbr.2009.0673